REMARKS

In the Office Action, the Examiner rejected claims 1-5 and 7-12 under 35 U.S.C. §102(b) as being anticipated by Horrobin et al. (US 6,245,811), rejected claims 1-13 under 35 U.S.C. §102(b) as being anticipated by Menard et al. (WO 02/09725), rejected claims 1-5 and 7-10 under 35 U.S.C. §102(b) as being anticipated by Horrobin et al. (US 5,603,959), and rejected claims 1-16 under 35 U.S.C. §103(a) as being obvious over Cook et al. (US 6,077,868) in view of Watkins et al. (Journal of the American College of Nutrition 2000, 19:478S-486S). Each rejection raised by the Examiner is addressed separately below. In view of the amendments noted above and the remarks below, applicants respectfully request reconsideration of the merits of this patent application.

No extension of time is believed to be necessary and no fee is believed to be due in connection with this response. However, if any extension of time is required in this or any subsequent response, please consider this to be a petition for the appropriate extension and a request to charge the petition fee to Deposit Account No. 17-0055. No other fee is believed to be due in connection with this response. However, if any fee is due in this or any subsequent response, please charge the fee to the same Deposit Account No. 17-0055.

Anticipation rejection under 35 U.S.C. §102 (b) based on Horrobin et al. (US 6,245,811)

The Examiner rejected claims 1-5 and 7-12 as being anticipated by Horrobin et al. (US 6,245,811). In particular, the Examiner alleged that the document disclosed a method for treating a disorder (rheumatoid arthritis) comprising administering to a patient in need thereof an effective amount of the compound according to claim 1 where R_1 is an acyl moiety corresponding to an acid (conjugated linoleic acid, CLA), citing column 14 at lines 55-62, column 15 at line 1, and claims 1, 10, and 28. Further, the Examiner asserts that said compound is an ester of conjugated linoleic acid. Applicants respectfully traverse the rejection in connection with the claims as amended. In this regard, applicants note that claim 1 has been amended to recite a composition that consists essentially of a conjugated linoleic acid and that Horrobin et al. (US 6,245,811) does not specifically disclose that conjugated linoleic acid can be used to treat rheumatoid arthritis.

Horrobin et al. (US 6,245,811) disclosed the treatment of rheumatoid arthritis in connection with the use of one or more of GLA, DGLA, SA and EPA but not CLA (see column 13, lines 12-16 and 30-31). The only activities of CLA disclosed by Horrobin et al. (US 6,245,811) are treating or preventing cancer, treating or preventing cardiovascular disease, treating metabolism diseases, promoting growth of protein-containing tissues, and acting as an antioxidant (see column 6, lines 35-39 and column 14, lines 6-10). The sections of Horrobin et al. (US 6,245,811) cited by the Examiner to raise the rejection, namely, column 14 at lines 55-62, column 15 at line 1, and claims 1, 10, and 28, list a plurality of compounds including among others GLA, DGLA, SA, EPA, and CLA for the treatment of a plurality of diseases including among others rheumatoid arthritis. A skilled artisan clearly understands from the overall disclosure of Horrobin et al. (US 6,245,811) that it is one or more of GLA, DGLA, SA and EPA, not CLA, in said compounds that make the compounds effective for treating rheumatoid arthritis. In other words, for any compound that is intended for use in treating rheumatoid arthritis, Horrobin et al. (US 6,245,811) teach that one or more of GLA, DGLA, SA and EPA should be included. As claim 1 has been amended to recite a composition consisting essentially of a conjugated linoleic acid and thus exclude other rheumatoid arthritis-treating compounds such as one or more of GLA, DGLA, SA and EPA, claim 1 and its dependents as amended are not anticipated by Horrobin et al. (US 6,245,811).

Anticipation rejection under 35 U.S.C. §102 (b) based on Menard et al. (WO 02/09725)

The Examiner rejected claims 1-13 as being anticipated by Menard et al. (WO 02/09725). In particular, the Examiner alleged that the document disclosed the treatment of degenerative joint diseases and rheumatoid arthritis by using a composition comprising conjugated linoleic acid (CLA), glucosamine and ascorbic acid. As discussed above, claim 1 has been amended to recite a composition consisting essentially of a conjugated linoleic acid and therefore limit the corresponding therapeutically active ingredient that can be included in the composition to only conjugated linoleic acid. Since Menard et al. specifically provide throughout the specification and claims that the treatment compositions disclosed therein require three active components, namely, conjugated linoleic acid, glucosamine and ascorbic acid, claim 1 and its dependents are not anticipated by Menard et al.

Anticipation rejection under 35 U.S.C. §102 (b) based on Horrobin et al. (US 5,603,959)

The Examiner rejected claims 1-5 and 7-10 as being anticipated by Horrobin et al. (US 5,603,959). In particular, the Examiner alleged that the document discloses a method of treating rheumatoid arthritis comprising administering to a person in need of such treatment an NSAID chemically linked to linoleic acid (LA). Applicants respectfully traverse the rejection.

While it is true that Horrobin et al. (US 5,603,959) disclose the use of an NSAID chemically linked to linoleic acid for treating rheumatoid arthritis, neither NSAID nor linoleic acid (LA) as disclosed by Horrobin et al. (US 5,603,959) is the same as conjugated linoleic acid (CLA). First of all, nowhere in Horrobin et al. (US 5,603,959) is it mentioned that conjugated linoleic acid is an NSAID. Secondly, it is well known in the art that LA (c9, c12-octadecadienoic acid) is an 18 carbon fatty acid with two double bonds at positions 9 and 12 in the orientation of c9, c12 and conjugated linoleic acid is a group of positional and geometrical isomers of linoleic acid with two double bonds typically at positions 9 and 11 or 10 and 12. *See e.g.*, paragraphs [0003]-[0005] of the present application; Li G. et al. at page 2134, right column, lines 7-9 (J. Lipid Res. 46:2134-2142, 2005, a copy of which is attached); and Yang M. et al. at page 51, right column, lines 1-4 (Exp. Biol. Med. 228:51-58, 2002, a copy of which is attached). Further, Li et al. show that CLA and LA can have different biological activities in that LA increases PEG production while CLA does not (Fig. 1C). Therefore, claims 1-5 and 7-10, which are directed at the use of conjugated linoleic acid, are not anticipated by Horrobin et al. (US 5,603,959).

Obviousness rejection under 35 U.S.C. §103 (a)

The Examiner rejected claims 1-16 as being obvious over Cook et al. (US 6,077,868) in view of Watkins et al. (Journal of the American College of Nutrition 2000, 19:478S-486S). In particular, the Examiner alleged that Cook et al. disclose a method of inhibiting cyclooxygenase 2 (COX-2) activity and reducing COX-2-mediated inflammation by using conjugated linoleic acid (CLA). Further, the Examiner alleged that Watkins et al. disclose that (1) anti-inflammatory diets are associated with decreased pathogenesis of rheumatoid arthritis, reduced inflammatory diseases and lowered cancer risk, (2) up-regulation of COX-2 contributes to inflammation, and (3) the beneficial anti-cancer effect of CLA is likely linked to down-regulation of COX-2

activity. In the Examiner's opinion, it would have been obvious to one of ordinary skill in the art, in view of the two references, to administer CLA for treating rheumatoid arthritis. In this regard, the Examiner asserts that one of ordinary skill in the art would be motivated to do the above, the practice of the invention of Cook et al. (US 6,077,868) would intrinsically treat rheumatoid arthritis, and there would be reasonable likelihood of success. Applicants respectfully traverse the rejection.

First of all, as the Examiner pointed out, Cook et al. do not expressly disclose that CLA can be used to treat a disease or condition (e.g., rheumatoid arthritis) caused by type III hypersensitivity. Watkins et al. do not cure this defect, at least from the perspective of reasonable likelihood of success. Watkins et al. is a review article on CLA and bone biology. It does not provide or discuss any data that establish the cause-effect relationship between COX-2 activity and rheumatoid arthritis. Rather, it reviewed the effect of CLA on the production of certain molecules such as PGE_2 (through inhibition of COX-2) and IL-6 that have only been implicated in rheumatic arthritis (see abstract and the paragraph bridging the left and right columns on page 483S). Without the cause-effect relationship established, the use of CLA as a COX-2 inhibitor for the treatment of rheumatoid arthritis would at most be something to try but there would not be any reasonable likelihood of success. In fact, Watkins et al. used uncertain languages in describing the use of CLA for the treatment of rheumatoid arthritis and inflammatory bone disease. For example, Watkins et al. stated that "[a]nti-inflammatory diets ... might help to reduce rheumatoid arthritis ..." (the abstract) and "one could hypothesize that ... the anti-inflammatory effects of CLA would be beneficial for the treatment of inflammatory bone disease" (the end of the paragraph bridging the left and right columns on page 483S). The fact that Watkins et al. mentioned in a speculative manner that the "anticancer effect of CLA is likely linked, in part, to down-regulation of COX-2 activity" adds nothing to support CLA's activity in rheumatoid arthritis treatment.

Secondly, there was evidence in the art at the time the present application was filed that CLA may aggravate rather than reduce rheumatoid arthritis. As discussed in the present application (e.g., paragraphs [00010] and [00011]), type III hypersensitivity is caused by immune complex deposition, leading to inflammatory reactions. In the case of rheumatoid arthritis, the immune complex is between the auto-antibodies to type II collagen and type II collagen. Yang et al. 2000 (Immunopharmacology and Immunotoxicology 22:433-449, copy attached) studied

the effect of CLA on another auto-antibody immune complex disease, systemic lupus erythematosus. As shown in Yang et al., CLA treatment promoted the earlier appearance of antinuclear antibodies as well as proteinuria (the first clinical sign of renal failure in Lupus due to antinuclear antibody immune complexes) in Lupus prone mice (Figs. 3 and 4). This would indicate that CLA may aggravate rather than reduce a disease such as rheumatoid arthritis that is caused by immune complex deposit.

With regard to the Examiner point that practicing of the invention of Cook et al. (US 6,077,868) would intrinsically treat rheumatoid arthritis, applicants respectfully note that the Examiner has not provided any evidence to establish that enhanced COX-2 activity is a cause of type III hypersensitivity diseases such as rheumatoid arthritis, much less that COX-2 is the sole cause such that inhibiting COX-2 would be sufficient to treat rheumatoid arthritis. In the case the Examiner made the statement based on personal knowledge, applicants respectfully request an affidavit from the Examiner under 37 CFR §1.104(d)(2).

For the above reasons, applicants respectfully submit that, at the time the present application was filed, the evidence from the art when considered as a whole do not provide the motivation for trying to use CLA to treat rheumatoid arthritis. Even for the sake of argument that one would try said treatment, there would not have been any reasonable likelihood of success for the same reasons.

Conclusion

Having addressed each rejection raised by the Examiner, the claims as amended are believed to be in condition for allowance and a Notice of Allowance is respectfully requested. Should any issues remain outstanding, the Examiner is invited to contact the undersigned at the telephone number appearing below if such would advance the prosecution of this application.

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10t,12c-conjugated linoleic acid inhibits lipopolysaccharide-induced cyclooxygenase expression in vitro and in vivo

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Abstract **Previous data demonstrated that conjugated linoleic acid (CLA) reduced eicosanoid release from select organs. We hypothesized that one active CLA isomer was responsible for the reduced prostaglandin release and that the mechanism was through the inhibition of inducible cyclooxygenase-2 (COX-2). Here, we examined the effects of 10t,12c-CLA and 9c,11t-CLA on COX-2 protein/mRNA expression, prostaglandin E_2 (PGE_2) production, and the mechanism by which CLA affects COX-2 expression and prostaglandin release. The COX-2 protein expression level was inhibited 80% by 10t, 12c-CLA and 26% by 9c,11t-CLA at 100 μM in vitro. PGE_2 production was decreased from 5.39 to 1.12 ng/2 × 10^6 cells by 10t,12c-CLA and from 5.7 to 4.5 ng/2 × 10^6 cells by 9c,11t-CLA at 100 μM. Mice fed 10t,12c-CLA but not 9c,11t-CLA were found to have a 34% decrease in COX-2 protein and a 43% reduction of PGE_2 release in the lung. 10t,12c-CLA reduced COX-2 mRNA expression level by 30% at 100 μM in vitro and by 30% in mouse lung in vivo. Reduced COX-2 mRNA was attributable to an inhibition of the nuclear factor κB (NF-κB) pathway by 10t,12c-CLA. These data suggested that the inhibition of NF-κB was one of the mechanisms for the reduced COX-2 expression and PGE_2 release by 10t,12c-CLA.**—Li, G., D. Barnes, D. Butz, D. Bjorling, and M. E. Cook. **10t,12c-conjugated linoleic acid inhibits lipopolysaccharide-induced cyclooxygenase expression in vitro and in vivo.** *J. Lipid Res.* **2005.** 46: **2134–2142.**

Supplementary key words inflammation • polyunsaturated fatty acid • prostaglandin • inducible nitric oxide synthase

Chronic inflammation is an essential step in the progression of many diseases, such as atherosclerosis (1), cancer (2), and neurodegenerative diseases (3). Inhibition of inflammation by targeting proinflammatory enzymes and cytokines has been repeatedly shown to be beneficial in the prevention and/or treatment of a broad range of diseases. Although novel synthetic inhibitors of proinflammatory enzymes and cytokines are effective in slowing the development of inflammatory diseases, side effects significantly limit their use (4). Hence, natural dietary substances that possess anti-inflammatory properties will be of potential value. Conjugated linoleic acid (CLA), a naturally occurring fatty acid found in ruminant animal fat, has been shown to be anticarcinogenic (5–7) and antiatherogenic (8–10). CLA is a collective term referring to a group of positional and geometrical isomers of *cis*-9,*cis*-12 linoleic acid (LA), of which 10t,12c-CLA and 9c,11t-CLA have received the most study. Of these two isomers, 9c,11t-CLA is the most abundant naturally occurring isomer, accounting for >75% of the total CLA in dairy products (11). However, although the 10t,12c-CLA content in food is low, the amount is biologically relevant (as low as 0.017 g of 10t,12t-CLA per 100 g of diet) (12).

CLA has also been reported to influence immune/inflammatory responses. Dietary CLA was shown to prevent body weight loss induced by lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α), or Sephadex (13–15) and to inhibit nitric oxide production and TNF-α release (16). Recent work has demonstrated that dietary CLA inhibited antigen-induced eicosanoid release in a type I hypersensitivity guinea pig model (17, 18). Mixed or purified isomers of CLA have also been demonstrated to inhibit inducible isoforms of cyclooxygenase (COX) mRNA and protein expression in vitro (19–21).

COX is the rate-limiting enzyme in the conversion of arachidonic acid to thromboxanes and prostaglandins. The enzyme exists in two isoforms, COX-1 and COX-2. COX-1 is constitutively expressed in most cell types and is thought to be responsible for the maintenance of gastrointestinal

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Abbreviations: CLA, conjugated linoleic acid; COX, cyclooxygenase; EIA, enzyme immunoassay; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1, interleukin-1; iNOS, inducible nitric oxide synthase; LA, linoleic acid; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; PGE_2, prostaglandin E_2; TNF-α, tumor necrosis factor-α.

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mucosa, kidney, platelet function, and other housekeeping functions. Although existing constitutively in some tissues, such as brain and kidney, COX-2 is a highly inducible isoform whose expression is regulated by many growth factors, LPS, and cytokines such as interleukin-1 (IL-1), IL-2, and TNF-α (22). Because COX-2 has been shown to play a significant role in many inflammatory diseases, it has been an important pharmacological target for the prevention and/or treatment of arthritis and cancer (23).

One pathway leading to the transcription of COX-2, along with other proinflammatory enzymes and cytokines, such as inducible nitric oxide synthase (iNOS) and TNF-α, is the nuclear factor κB (NF-κB)-dependent pathway (24). NF-κB is a multiple-subunit transcription factor whose activation is involved in carcinogenesis, atherosclerosis, and rheumatoid arthritis (25, 26). Currently, NF-κB family members include p50/p105, p52/p100, c-Rel, RelB, and p65. In resting cells, NF-κB is sequestered in the cytoplasm by the inhibitory proteins IκBα, IκBβ, and IκBε. Upon stimulation by cytokines or LPS, IκBα is phosphorylated and then degraded in a ubiquitin-proteosome-dependent manner. NF-κB is liberated and translocates to the nucleus, where it activates the gene transcription of proinflammatory mediators. Thus, NF-κB represents a central mediator in inflammatory responses (25, 26). It has been demonstrated that nonsteroidal anti-inflammatory drugs such as aspirin (27), steroidal anti-inflammatory drugs such as dexamethasone (28, 29), and fatty acids such as docosahexaenoic acid and eicosapentenoic acid (30) exert anti-inflammatory responses by inhibiting the activation of NF-κB.

Based on the previous results with CLA, this study was designed to investigate the isomer-specific effect of CLA on COX-2 mRNA and/or protein inhibition and whether reduced COX-2 protein expression was attributable to the inhibitory effects on the NF-κB pathway. We found that 10t,12c-CLA was the isomer that inhibited COX-2 protein, mRNA level, and prostaglandin E_2 (PGE_2) release both in vitro and in vivo. The other isomer, 9c,11t-CLA, was only effective at 100 μM in vitro and was not effective in vivo. The 10t,12c-CLA isomer was also found to inhibit IκBα phosphorylation and to reduce the activated level of NF-κB binding to its consensus site in DNA, suggesting an NF-κB-dependent molecular mechanism.

MATERIALS AND METHODS

Materials

LA (>99%) and 10t,12c-CLA and 9c,11t-CLA isomers (>98%) for in vitro studies were purchased from Matreya, Inc. (Pleasant Gap, PA). CLA isomers for feeding trials were from Natural Lipid, Inc. (Hovdebygda, Norway). The composition of the Natural Lipid 10t,12c-CLA isomer was 0.4% oleic acid, 3.7% 9c,11t-CLA, 92.5% 10t,12c-CLA, and ~3.4% other fatty acids. The composition of the Natural Lipid 9c,11t-CLA isomer was 4.5% oleic acid, 90.3% 9c,11t-CLA, 2.4% 10t,12c-CLA, and 2.8% other fatty acids.

The PGE_2 enzyme immunoassay (EIA) kit and COX-2 antibody were purchased from Cayman Chemical (Ann Arbor, MI). IκBα and phosphorylated IκBα antibodies were purchased from Cell Signaling Technology (Beverly, MA). The TransAM NF-κB DNA binding ELISA kit was from Active Motif (Carlsbad, CA). Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and β-actin antibodies were from Trevigen (Gaithersburg, MD) and Sigma (St. Louis, MO), respectively. LPS (*Escherichia coli* O55:B5) was from Sigma. Premium FBS was from Biowhittaker (Walkersville, MD). LR White resin was from London Resin Co. Ltd. (Berkshire, UK). Alexa Fluor 488 goat anti-rabbit IgG was from Molecular Probes (Eugene, OR). BALB/c mice were from Jackson Laboratory (Bar Harbor, ME).

Cell culture

The Raw264.7 macrophage cell line was maintained in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO_2 incubator. Cells (1×10^6/well) were plated onto 24-well plates. When cells were confluent, free fatty acids dissolved in ethanol, LA, 10t,12c-CLA, 9c,11t-CLA (3.3, 10, 33, and 100 μM), or ethanol were added to DMEM containing 0.5% FBS. Twenty-four hours later, LPS (100 ng/ml or 1 μg/ml) was introduced into the cell culture for various lengths of time. Cell viability was >96% in LA- and CLA-treated cells, and total protein concentration was not affected by LA or CLA treatment (data not shown). The highest ethanol concentration in each sample was <0.19%.

Western blot

Confluent Raw264.7 macrophages were pretreated with CLA isomers for 24 h and then stimulated with LPS for the indicated time for each experiment. Cells were then lysed with radioimmunoprecipitation assay buffer containing proteinase and phosphatase inhibitors (1 mM phenylmethylsulfonyl, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 mM Na_3VO_4, and 1 mM NaF). Protein concentration was measured by the Bradford assay (31). Denatured proteins were separated with 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane. After nonspecific blocking, membranes were incubated with primary COX-2, IκBα, or phosphorylated IκBα antibody and then with a horseradish peroxidase-coupled secondary antibody before being exposed to Fuji medical X-ray film. A preliminary study was conducted to optimize the linear relationship between the band area and the amount of loaded protein. For COX-2, percentage of control was calculated as % control = (COX-2 band area with fatty acids/COX-2 band area with ethanol) × 100. Cell culture medium for the analysis of COX-2 was also collected for PGE_2 release analysis. For IκBα and phosphorylated IκBα, % positive control = (band area in fatty acids and LPS-treated sample/band area in ethanol- and LPS-treated sample) × 100. Lung tissue was ground in liquid nitrogen, and ~100 μg of powder was dissolved in 300 μl of radioimmunoprecipitation assay buffer with proteinase and phosphatase inhibitors as described above. The powder was further homogenized in the ice bath with an Ultra-turrax homogenizer and then centrifuged at 10,000 *g* for 10 min. The protein concentration in the supernatant was measured by the Bradford assay (31), and Western blotting was conducted as described above. G3PDH or β-actin was also measured as a loading control. The supernatant was also used for PGE_2 analysis.

PGE_2 analysis

PGE_2 was determined using EIA kits obtained from Cayman Chemical. Procedures were followed as indicated in the kit instructions.

Animal treatment

Animal experimentation was approved by the Animal Care Committee of the University of Wisconsin-Madison College of Agricul-

tural and Life Sciences. Two separate feeding trials were conducted to examine the isomer-specific effects of CLA on COX-2 mRNA and protein level. For each trial, 24 BALB/c male mice (4 weeks old) were randomly assigned to four groups: Corn Oil (CO), CO + LPS, 9c,11t-CLA + LPS, and 10t,12c-CLA + LPS. The mice were fed a basal diet (99% semipurified diet + 1% CO) for at least 2 days and then placed on their respective diet treatment for at least 21 days. Diet composition is shown in **Table 1**. At the end of feeding, mice were injected intraperitoneally with LPS (1 mg/kg body weight) or PBS (vehicle). The mice were euthanized with CO_2 after LPS injection, and lungs were dissected and snap-frozen for mRNA isolation and Western blot or placed in PBS for immunocytochemical analysis of COX-2. PGE_2 production from lung homogenates was measured by EIA.

Immunocytochemistry

The procedure followed has been described elsewhere (32). Briefly, the lung was rinsed with PBS, fixed in 3% paraformaldehyde for 1.5 h, washed in 0.1 M glycine in PBS, dehydrated with graded concentrations of ethanol, and incubated with LR White resin. Sections (1 μm thick) were blocked with 5% BSA and then incubated with COX-2 antibody for 1.5 h. Bound antibodies were detected with fluorescent secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG incubated for 1.5 h). Sections were washed with PBS three times and then stored at 4°C until observation by light microscopy with the appropriate filter settings (AxioCam HRm, HBO 100).

Isolation of mRNA and RT-PCR

Confluent cells were treated with CLA isomers for 24 h and then incubated with LPS for 4 h (COX-2) or 6 h (iNOS). mRNA was isolated according to procedures described by Diaz, Crenshaw, and Wiltbank (33). Briefly, cells were dissolved in 100 μl of lysis buffer (4 M guanidium isothiocyanate, 0.5% sarcosyl, 10 mM Tris-HCl, pH 8.0, and 1% dithiothreitol) and passed through a 25 gauge needle to shear DNA. Binding buffer (200 μl; 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 400 mM NaCl) was added, and samples were centrifuged. Supernatants were incubated with Magnetight™ oligo(dT) magnetic particles for at least 5 min. The particles were washed four times with wash buffer (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA), and the associated mRNA was rinsed off the beads with 10 μl of elution buffer (2 mM EDTA). Isolated mRNA (2 μl) was reverse-transcribed in the master mix (4.0 μl of reaction buffer, 1 μl of 100 μM random primer, 0.4 μl of 10 mM deoxynucleotide triphosphate, 0.2 μl of reverse transcriptase, and 12.4 μl of water). Polymerase chain reaction was used to amplify individual genes using specific primers. mRNA for the G3PDH gene was amplified as an internal control. Their respective primers were as follows: COX-2 upstream, 5′-CAA-GCA-GTG-GCA-AGG-CCT-CCA-3′; COX-2 downstream, 5′-GGC-ACT-TGC-ATT-GAT-GGT-GGC-T-3′; iNOS upstream, 5′-GGC-TTG-CCC-CTG-GAA-GTT-TCT-CTT-CAA-AGT-C-3′; iNOS downstream, 5′-AAG-GAG-CCA-TAA-TAC-TGG-TTG-ATG-3′; G3PDH upstream, 5′-GGC-ATT-CTC-GGC-TAC-ACT-GA-3′; G3PDH downstream, 5′-CAT-ACC-AGG-AAA-TTA-GCT-TGA-C-3′.

Samples from mouse lung were homogenized in lysis buffer solution, and mRNA was then similarly isolated and amplified as those from Raw264.7 macrophages. Amplification products were separated with 5% PAGE. Band area was analyzed by Quantity One software. Band area was shown to be proportional to the amount of cDNA within the amplification cycles. For mRNA level from Raw264.7 macrophages, percentage control was derived by first normalizing the band area of the amplification product to the band area of its corresponding G3PDH, then dividing the results by the ratio obtained from the positive control and multiplying by 100. mRNA level from lung tissue were directly normalized with its corresponding area of G3PDH.

Determination of NF-κB binding by DNA binding ELISA

Confluent Raw264.7 macrophages were incubated with LA or 10t,12c-CLA (50 or 100 μM) in DMEM containing no FBS. After 24 h, cells were incubated with 1 μg/ml LPS for 4 h. Cells were then harvested for the determination of NF-κB (P50 and P65) binding by ELISA according to the instructions in the TransAM NF-κB DNA binding ELISA kit. In brief, activated P65 and/or P50 binds to its consensus site (5′-GGGACTTTCC-3′) immobilized on the wells. The primary antibody of P65 or P50 recognizes and binds to p65 or p50 only when it is activated and bound to its consensus site. HRP-conjugated secondary antibody converts its substrates for the final determination by spectrophotometry.

Statistical analysis

Data were analyzed with Student's *t*-test. Differences were considered significant at $P < 0.05$.

RESULTS

Effect of 10t,12c-CLA and 9c,11t-CLA on COX-2 protein expression and PGE_2 production in vitro

COX-2 protein was not detectable in the resting Raw264.7 macrophages. Upon stimulation by LPS, COX-2 protein

TABLE 1. Diet composition

	Experimental Groups			
Ingredient (g/100 g)	CO	CO + LPS	10t,12c-CLA + LPS	9c,11t-CLA + LPS
Sucrose	47.6	47.6	47.6	47.6
Casein	21	21	21	21
Corn starch	15	15	15	15
Corn oil	6	6	5.75	5.75
Cellulose	5	5	5	5
AIN-76 mineral mix	3.5	3.5	3.5	3.5
AIN-76 vitamin mix	1	1	1	1
Calcium carbonate	0.4	0.4	0.4	0.4
DL-Methionine	0.3	0.3	0.3	0.3
Choline bitartrate	0.2	0. 2	0.2	0.2
Ethoxyquin	0.001	0.001	0.001	0.001
10t,12c-CLA	—	—	0.25	—
9c,11t-CLA	—	—	—	0.25

CLA, conjugated linoleic acid; LPS, lipopolysaccharide.

was significantly induced. After Raw264.7 macrophages were incubated with LA, 10t,12c-CLA, or 9c,11t-CLA isomers for 24 h and stimulated with LPS (100 ng/ml) for 8 h, cell viability (assessed by trypan blue exclusion) exceeded 96%. 10t,12c-CLA decreased COX-2 protein expression significantly. When macrophages were treated with 100 μM 10t,12c-CLA, COX-2 protein levels were only ~21.6% of the control values (0.19% ethanol + LPS). 9c,11t-CLA also significantly inhibited COX-2 protein expression, decreasing expression to 74.3% of control levels at 100 μM. However, LA did not inhibit COX-2 protein expression even at 100 μM (**Fig. 1A, B**).

LPS-induced PGE_2 production by Raw264.7 macrophages in the presence of LA, 10t,12c-CLA, or 9c,11t-CLA isomers was also measured (Fig. 1C). It was found that PGE_2 release by macrophages treated with 10t,12c-CLA was inhibited in a concentration-dependent manner. PGE_2 release by cells treated with 0, 3.3, 10, 33, or 100 μM 10t,12c-CLA was 5.39, 4.65, 3.28, 2.83, or 1.12 ng/2 × 10^6 cells, respectively. 9c,11t-CLA decreased PGE_2 production only at 100 μM (PGE_2 production was reduced from 5.7 to 4.5 ng/2 × 10^6 cells). In contrast, PGE_2 production was dose-dependently increased by LA and was 5.8, 5.7, 7.3, 8.1, and 11.9 ng/2 × 10^6 cells for 0, 3.3, 10, 33, and 100 μM LA (Fig. 1C).

Effect of 10t,12c-CLA and 9c,11t-CLA on COX-2 protein expression and PGE_2 production in vivo

To test whether COX-2 expression and PGE_2 production were also decreased in vivo, mice were fed purified CLA isomers. In a preliminary trial, COX-2 protein expression was detected in the lung (**Fig. 2A**) but not in the spleen (data not shown) 8 h after mice were injected with LPS. Although 9c,11t-CLA had no effect on LPS-induced COX-2 protein expression in the lung, 10t,12c-CLA reduced COX-2 protein expression relative to the control diet-fed mice (Fig. 2A, B). The LPS-induced increase of PGE_2 release from lung was reduced in 10t,12c-CLA-fed mice but not in 9c,11t-CLA-fed mice relative to LPS-injected mice fed the control diet (Fig. 2C). The increased COX-2 protein expression in the lung attributable to LPS injection and inhibition of the expression by feeding 10t,12c-CLA but not 9c,11t-CLA is shown in Fig. 2D using immunocytochemistry.

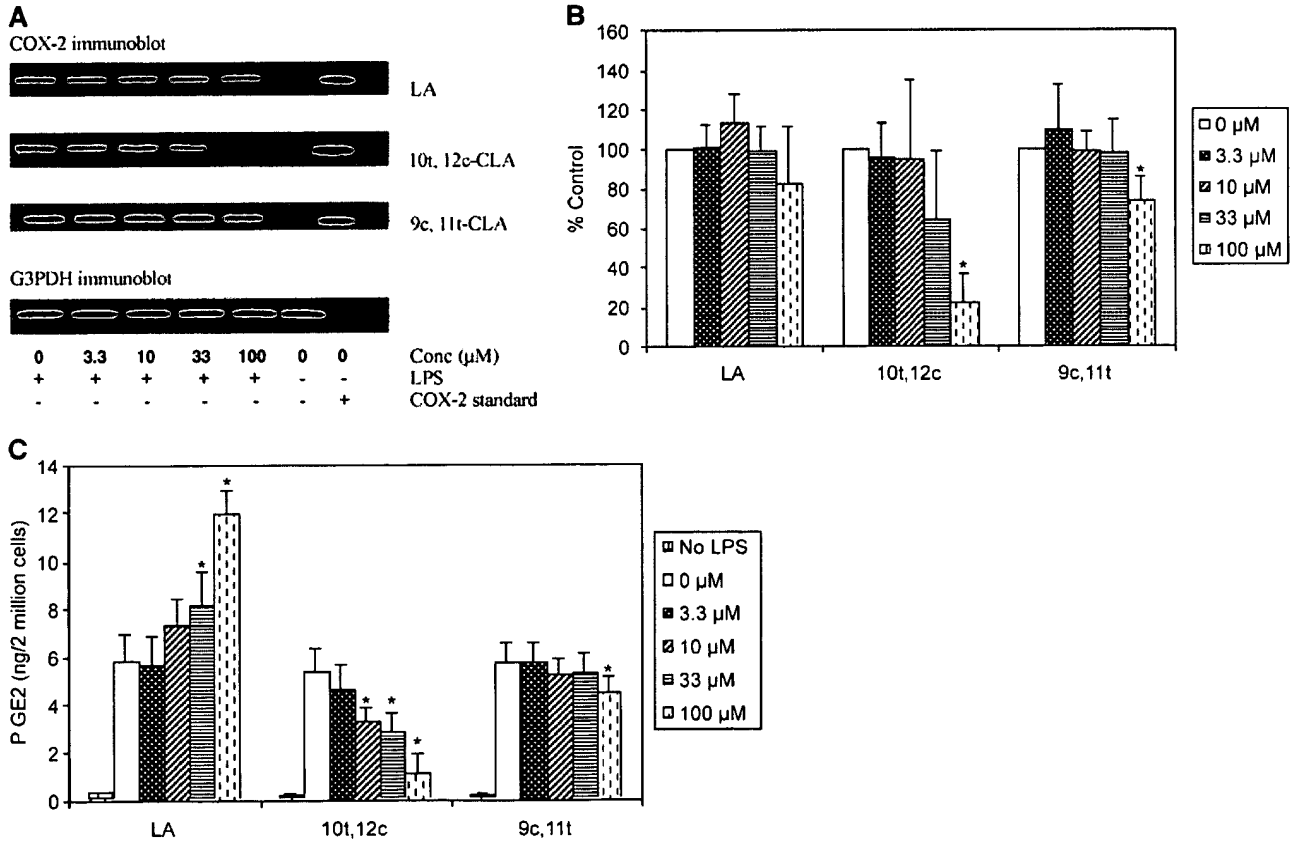

Fig. 1. Effects of 10t,12c-CLA and 9c,11t-CLA on cyclooxygenase-2 (COX-2) protein expression (A, B) and prostaglandin E_2 (PGE_2) production (C) in Raw264.7 macrophages. Confluent Raw264.7 macrophages were treated with different concentrations of linoleic acid (LA), 10t,12c-CLA, or 9c,11t-CLA (0, 3.3, 10, 33, and 100 μM) or an ethanol solution (control) for 24 h in 0.5% FBS DMEM. Lipopolysaccharide (LPS; 100 ng/ml) was introduced for 8 h to induce the expression of COX-2. The cell lysate and cell medium were harvested to determine the expression of COX-2 and PGE_2 production. Data are presented as means ± SD from four replicates. * $P < 0.05$ versus control.

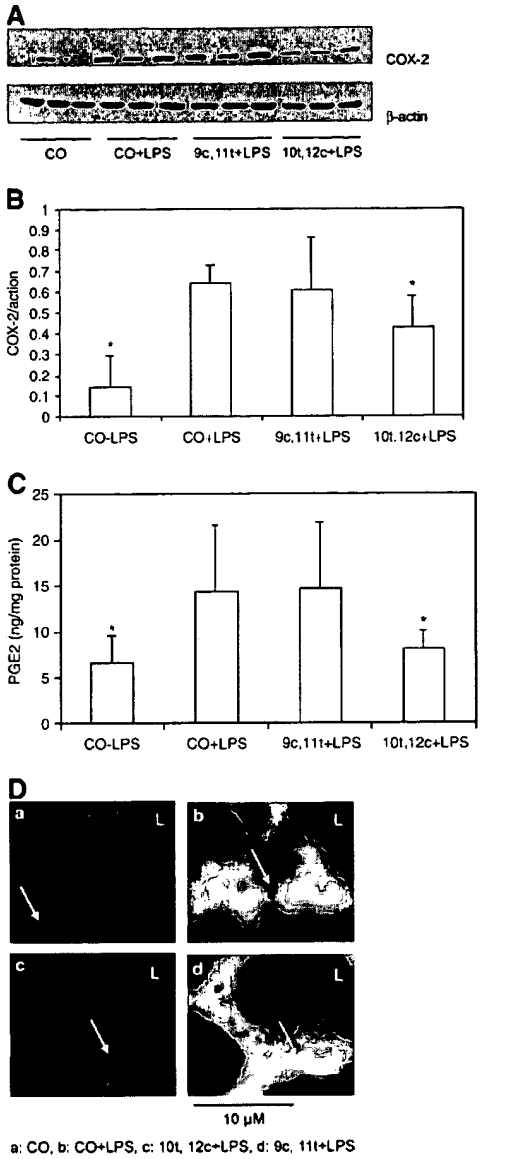

Fig. 2. Inhibitory effects of 10t,12c-CLA on COX-2 protein expression (A, B, D) and PGE_2 release (C) in mouse lung. Twenty-four BALB/c male mice (4 weeks old) were randomly assigned to four groups: CO, CO + LPS, 9c,11t-CLA + LPS, and 10t,12c-CLA + LPS. The mice were fed a basal diet (99% semipurified diet + 1% CO) for 11 days and then placed on their respective diet treatment for 26 days. On the 26th day, mice were injected with LPS (1 mg/kg) or vehicle. Mice were euthanized with CO_2 at 8 h after LPS injection, and lungs were harvested for Western blot analysis of COX-2. Each band represents data from one mouse (A), and PGE_2 concentration was determined by enzyme immunoassay (C). Data are presented as means ± SD, n = 6 per group (data from three animals in each treatment are shown). * $P < 0.05$ compared with the CO + LPS group by one-tailed *t*-test. D: After the mice were euthanized with CO_2 at 8 h after LPS injection, lungs were taken out for immunocytochemical analysis. The immunocytochemistry procedures followed are described in Materials and Methods. Fluorescence was detected with a microscope, and fluorescence intensity (shown as white) reflects the abundance of COX-2 in the cell. The small black areas inside the bright cytoplasm is the nucleus of the cells (arrows), and the large black area surrounding the bright cytoplasm is the lumen of the lung (L).

Effect of 10t,12c-CLA on COX-2 mRNA in vitro and in vivo and on iNOS mRNA in vitro

One pathway by which LPS induces COX-2 expression is through NF-κB activation. If reduced COX-2 protein expression was attributable to the inhibition of the NF-κB pathway, the COX-2 mRNA level should also be inhibited by 10t,12c-CLA. Raw264.7 macrophages treated with 10t,12c-CLA had reduced COX-2 mRNA expression (**Fig. 3A**). Likewise, in a feeding trial, mice fed 10t,12c-CLA had decreased COX-2 mRNA by 29.4% ($P = 0.059$; Fig. 3B). Because gene transcription of iNOS has been shown to be under the regulation of NF-κB, the effects of 10t,12c-CLA on iNOS mRNA were measured. It was shown that iNOS mRNA was reduced by 26% in the presence of 100 μM 10t,12c-CLA isomer (Fig. 3C).

Effect of 10t,12c-CLA on the NF-κB-mediated signal transduction pathway

NF-κB is a transcription factor that binds to inhibitory proteins such as IκBα, IκBβ, and IκBε in the cytoplasm of resting cells. Upon activation by LPS, cytokines, or growth factors, IκBα is phosphorylated and then degraded. NF-κB then translocates to the nucleus and activates the transcription of various proinflammatory cytokines and enzymes (COX-2 and iNOS). Phosphorylation of IκBα represents an important step for the activation of NF-κB in initiating the transcription of inflammatory mediators.

After incubation of Raw264.7 macrophages with 10t,12c-CLA, cells were stimulated with LPS for 4 h. As shown in **Fig. 4A**, phosphorylation of IκBα was inhibited by 10t,12c-CLA, but not by LA, in a concentration-dependent manner. Total IκBα was also determined in cells treated with LA or 10t,12c-CLA and stimulated with LPS for 4 h. Both compounds were shown to have no effect on the total level of IκBα (Fig. 4B).

LPS in the absence of supplemental fatty acids increased the binding of two NF-κB member proteins, P50 and P65, to the DNA consensus sites (Fig. 4C, D). 10t,12c-CLA, but not LA, was shown to decrease the binding of P50 and P65 to the consensus site (Fig. 4C, D).

DISCUSSION

CLA has been shown to prevent immune-induced wasting (13–15), decrease antigen-induced eicosanoid release (17, 18), and inhibit carcinogenesis (5–7) and atherosclerosis (8–10) in several animal models. The multiple functionalities of CLA have been hypothesized to be related to eicosanoid signaling (14). In the present study, the isomer of CLA that downregulates COX-2 protein and mRNA expression in vitro and in vivo was 10t,12c-CLA (active isomer). The data presented here support the hypothesis that 10t,12c-CLA exerts its protective effect against inflammatory diseases through the inhibition of COX-2 protein expression and activity.

This study first examined the effects of these fatty acids on LPS-induced COX-2 expression and PGE_2 production in a macrophage cell line. The data demonstrated that after LPS treatment, COX-2 protein expression and PGE_2 release by

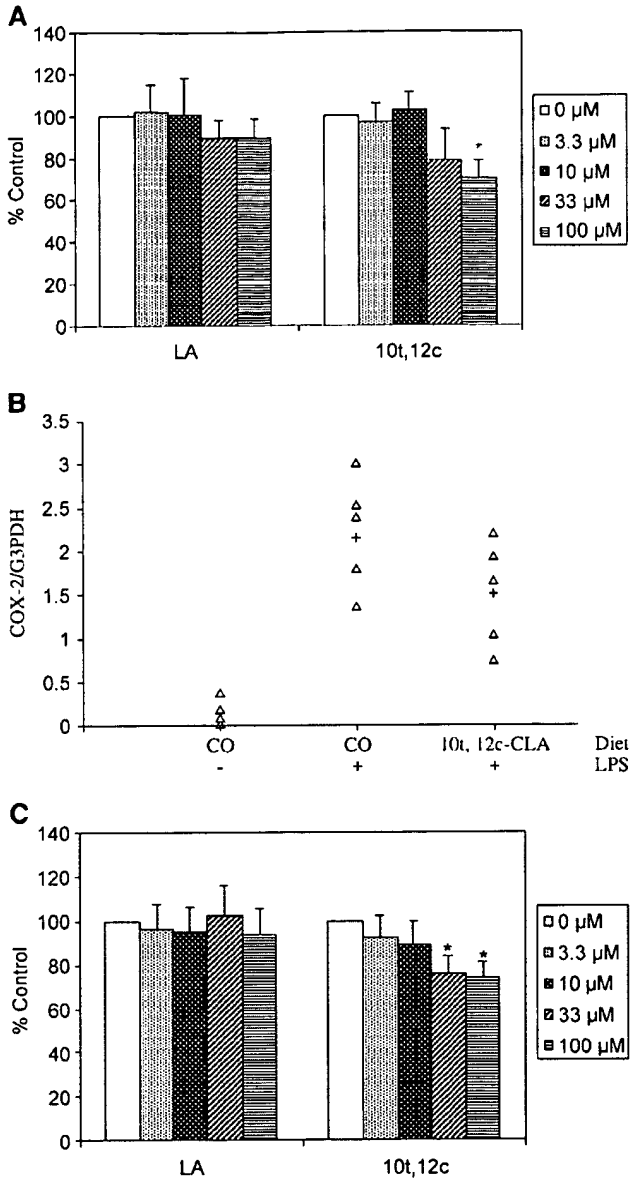

Fig. 3. Effects of LA and 10t,12c-CLA on LPS-induced COX-2 mRNA in vitro (A) and in vivo (B) and on inducible nitric oxide synthase (iNOS) mRNA in vitro (C). A, C: Confluent Raw264.7 macrophages were incubated with LA, 10t,12c-CLA, or 9c,11t-CLA (0, 3.3, 10, 33, and 100 μM) or ethanol (positive control) for 24 h and then were stimulated with LPS for 4 h (COX-2) or 6 h (iNOS) based on a kinetic study. B: Twenty-four BALB/c male mice (4 weeks old) were randomly assigned to four groups: CO, CO + LPS, 9c,11t-CLA + LPS, and 10t,12c-CLA + LPS. The mice were fed a basal diet (99% semipurified diet + 1% CO) for 2 days and then placed on their respective diet treatment for 21 days. On the 22nd day, mice were injected with LPS (1 mg/kg) or vehicle. G3PDH, glyceraldehyde-3-phosphate dehydrogenase. Mice were euthanized with CO_2 at 2 h after LPS injection, and lungs were harvested for mRNA isolation. There are five samples in the 10t,12c group because of an insufficient amount of samples from the sixth mouse. The plus sign represents the average value in each group. Isolation of mRNA, reverse transcription-polymerase chain reaction, and percentage control calculation were performed as described in Materials and Methods. Data are presented as means ± SD of five or six replicates. * $P < 0.05$ versus control.

LPS in macrophages were increased, as shown in previous studies (30, 34). The increase of COX-2 protein and PGE_2 release was inhibited by 10t,12c-CLA in a concentration-dependent manner and by 9c,11t-CLA only at 100 μM (Fig. 1A, B). Although 10t,12c-CLA was shown to reduce COX-2 protein expression and PGE_2 release, the concentration to reduce protein expression was higher than that required to decrease PGE_2 release. CLA has been shown to be a direct inhibitor of COX-2 enzymatic activity (17, 35; unpublished data) and to reduce arachidonic acid level in phospholipids through the inhibition of fatty acid elongase (36). Hence, 10t,12c-CLA-induced decreases in COX-2 product formation could be the results of *1*) decreased COX-2 protein, *2*) decreased substrate availability, and *3*) decreased COX-2 activity.

It has been shown that fatty acids inhibit COX-2 protein expression (30) and COX enzymatic activity (37) in vitro, but the in vivo effects of CLA isomers have not been shown. Feeding trials were conducted to investigate the effects of 9c,11t-CLA and 10t,12c-CLA isomers on COX-2 protein expression and PGE_2 release in vivo. Mouse lung was selected based on known COX-2 expression and PGE_2 release from this organ after inflammatory stimulation (18, 38–40). As shown in Fig. 2, feeding 10t,12c-CLA, but not 9c,11t-CLA, significantly reduced LPS-induced COX-2 protein expression and PGE_2 release compared with that in CO-fed LPS-injected mice. Although it has been reported that mixed isomers of CLA inhibited COX-2 expression and PGE_2 release in vitro (20, 21), this is the first study of the isomer-specific effects of CLA on COX-2 protein expression and PGE_2 release both in vitro and in vivo.

COX-2 expression is regulated at the transcriptional and posttranscriptional levels (see below). To identify whether reduced COX-2 protein by 10t,12c-CLA was attributable to its reduced mRNA level, the effects of 10t,12c-CLA in vitro (macrophage) and in vivo (mouse lung) on LPS-induced COX-2 mRNA were studied. As shown in Fig. 3, COX-2 and iNOS mRNA levels were also decreased by the 10t,12c-CLA in macrophages. COX-2 mRNA was also decreased in lungs of 10t,12c-CLA-fed mice. Interestingly, 10t,12c-CLA inhibited COX-2 protein by 80% and mRNA by only 30% in macrophages. Several pharmacological agents have been shown to participate in the posttranscriptional regulation of COX-2 mRNA, such as dexamethasone (41) and leptomycin B (42). Pyrrolidine dithiocarbamate, an oxidant scavenger, and rotenone, an inhibitor of the mitochondrial electron transport system, have also been reported to inhibit the expression of COX-2 protein expression without affecting COX-2 mRNA level in primary mesangial cell cultures (43). The differential inhibition rate on COX-2 mRNA and protein expression by 10t,12c-CLA may be attributed to the posttranscriptional regulation of the gene product. Although there are no data for 10t,12c-CLA involved in the posttranscriptional regulation of COX-2, polyunsaturated fatty acids have been shown to posttranscriptionally regulate for Δ-9 desaturase-1, glucose transporter-4, and Δ-9 desaturase-2 in adipocytes and lymphocytes (44).

It is well documented that LPS stimulation leads to the activation of NF-κB (45). When cells are not activated, NF-κB is sequestered in the cytoplasm by the inhibitory proteins

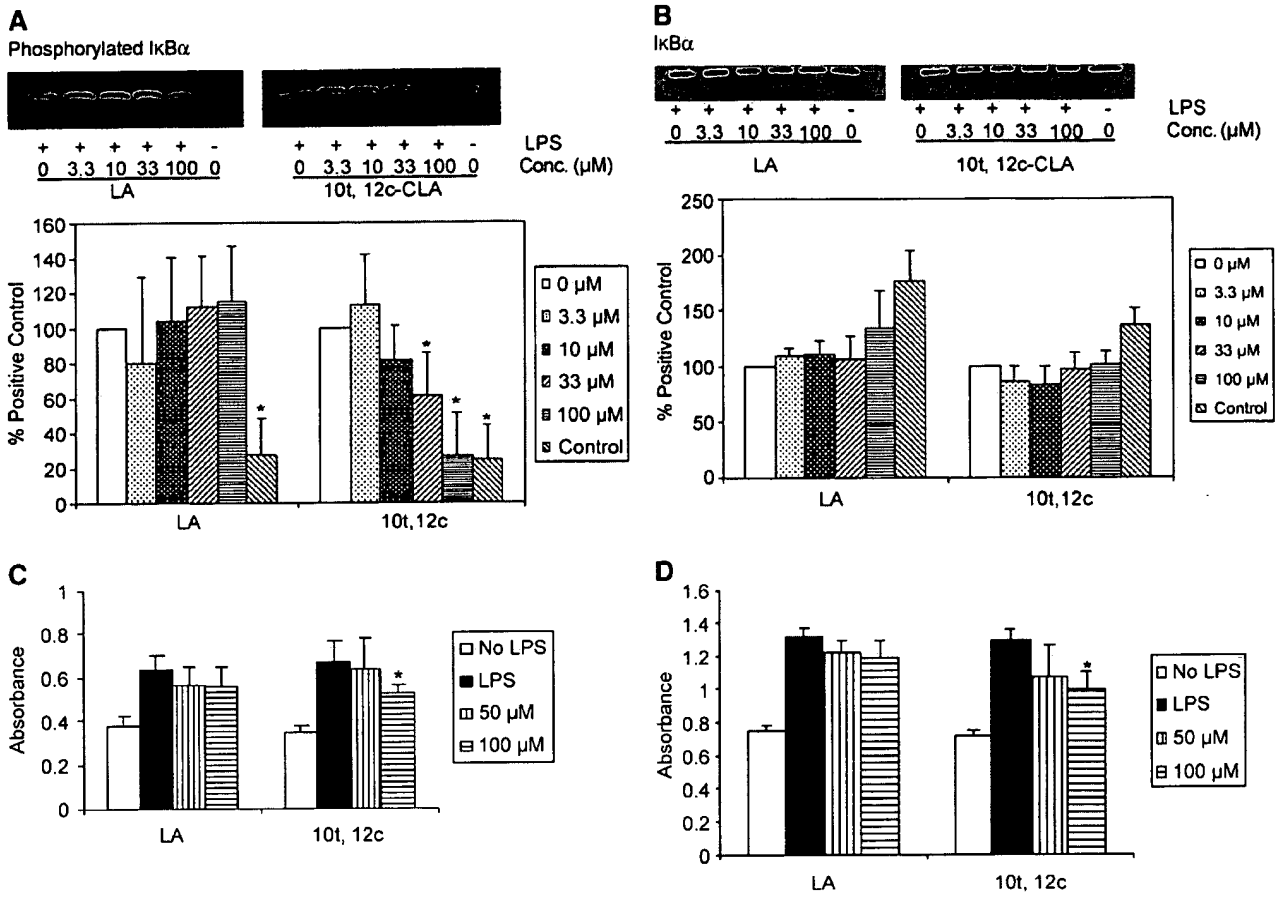

Fig. 4. Effect of 10t,12c-CLA on IκBα phosphorylation (A), total IκBα level (B), nuclear factor κB (NF-κB) p50 binding (C), and NF-κB p65 binding (D) in Raw264.7 macrophages. A, B: The cells were incubated with different concentrations (0, 3.3, 10, 33, and 100 μM) of LA, 10t,12c-CLA, or ethanol for 24 h and then were stimulated with LPS (1 μg/ml) for 4 h. Cell lysate was separated with SDS-PAGE and then probed with phosphorylated IκBα or IκBα antibody. Data are presented as means ± SD of four replicates. * $P < 0.05$ versus a positive control (ethanol + LPS). Percentage positive control = (band area in fatty acids and LPS-treated sample/band area in ethanol- and LPS-treated sample) × 100. C, D: The cells were incubated with different concentrations (50 and 100 μM) of LA, 10t,12c-CLA, or ethanol for 24 h and then were stimulated with LPS (1 μg/ml) for 4 h. Cell lysate was harvested for the determination of p50 and p65 binding according to kit instruction. Data are presented as means ± SD of four (p50) or five (p65) replicates. * $P < 0.05$ versus a positive control (ethanol + LPS).

IκBα, IκBβ, and IκBε. Upon stimulation by growth factors, cytokines, or LPS, IκBα is phosphorylated and then degraded in a ubiquitin-proteosome-dependent manner. Liberated NF-κB translocates to the nucleus and activates the gene transcription of proinflammatory mediators. NF-κB thus is an important transcription factor in the regulation of proinflammatory enzymes and cytokines (26). Pharmaceutical strategies for the prevention of inflammatory diseases and cancer have been designed to target NF-κB (46). Decreased COX-2 and iNOS mRNA was at least partially attributable to the inhibition of the NF-κB-mediated signal transduction pathway. As shown in Fig. 4, 10t,12c-CLA inhibited IκBα phosphorylation after LPS stimulation for 4 h in a concentration-dependent manner, whereas LA had no inhibitory effects on IκBα phosphorylation (Fig. 4A). Inhibition of IκBα phosphorylation would thus inhibit the translocation of NF-κB to the nucleus and subsequent gene transcription under NF-κB regulation. Indeed, the treatment of 10t,12c-CLA significantly decreased the binding of activated p50 and p65 to its consensus site (Fig. 4C, D). These data suggested that the decreased binding of activated p50 and p65 was at least partially attributable to the inhibition of IκBα phosphorylation. Inhibition of IκBα phosphorylation has also been the proposed mechanism by which n-3 fatty acids reduce TNF-α release from macrophages (47). Interestingly, the total level of IκBα was not affected by LA and 10t,12c-CLA (Fig. 4B), similar to what has been observed with the effects of n-3 fatty acids on IκBα level in macrophages (47). Other NF-κB-regulated products, such as TNF-α and IL-1β, are also affected by CLA. Yang and Cook (16) have demonstrated that CLA reduced TNF-α release in vitro and ex vivo from CLA-treated macrophages and mice, respectively. In another study, 10t,12c-CLA-fed mice were shown to exhibit lower plasma TNF-α compared with CO-fed mice (unpublished data). CLA was also shown to decrease basal and LPS-stimulated IL-1 levels in macrophages of rats fed a diet containing CLA (48). Although the proteins of TNF-α and IL-

1β were shown to be affected by CLA in these studies, its effects on mRNA level warrant further examination. What is not clear from this and other studies is whether the effects of 10t,12c-CLA on IκBα phosphorylation are direct or are the result of an event upstream of IκBα phosphorylation.

One major finding in the current study is that 10t,12c-CLA, a naturally occurring fatty acid, inhibits COX-2 and PGE_2 in vivo. Decreased COX-2 protein level and PGE_2 production in vivo may help explain some beneficial effects of CLA in a number of animal models, such as its inhibitory effects on carcinogenesis (5–7), atherosclerosis (8–10), and other diseases. Because COX-2 has also been reported to play an important role in the development of systemic lupus erythematosus (49, 50), the prolonged survival after onset of proteinuria could be related to the COX-2 protein and PGE_2 inhibition in mice fed a CLA diet (16, 51). Interestingly, it was shown the CLA feeding spared gastrocnemius muscle breakdown in a mouse cancer cachexia model that is also sensitive to COX-2 inhibition (52). Given the role of COX-2 in inflammatory diseases and 10t,12c-CLA's effect on COX-2 and other proinflammatory mediators, it is expected that a diet rich in CLA may help prevent an array of inflammatory diseases.

In summary, this study has shown that 10t,12c-CLA is the active isomer in inhibiting COX-2 protein expression and PGE_2 production in both in vitro and in vivo models. COX-2 inhibition was probably mediated at both transcriptional and posttranscriptional levels. Interference with NF-κB activation was one of the potential mechanisms underlying the isomer's effect in decreasing COX-2 and other cytokine gene transcription. These results suggest that some of CLA's beneficial effects may be mediated through inhibitory effects on COX-2 activity/protein expression, and 10t,12c-CLA may represent a naturally occurring nutrient against inflammatory diseases in which COX-2 is significantly involved.

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DIETARY CONJUGATED LINOLEIC ACID PROTECTS AGAINST END STAGE DISEASE OF SYSTEMIC LUPUS ERYTHEMATOSUS IN THE NZB/W F1 MOUSE

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Abstract

Conjugated linoleic acid (CLA) is a naturally occurring fatty acid with anti-carcinogenic, anti-atherosclerotic and immune-enhancing activities. Dietary CLA accelerated the onset of proteinuria in autoimmune-prone NZB/W F1 mice but did not affect anti-DNA antibody production. Body weight of the CLA group was decreased compared to the control group at the time proteinuria first developed. CLA group also had slightly earlier mortality than control fed mice, however the mean days of survival did not differ between CLA and control fed mice. Body weight loss between proteinuria onset and death was approximately twice as much in the control group as in the CLA group. Moreover, duration between proteinuria and death was longer in the CLA than in the control group. Our data suggested that dietary CLA may accelerate the autoimmune symptoms of NZB/W F1 mice, however, CLA protected against the disease related body weight loss and prolonged survival after proteinuria.

INTRODUCTION

Conjugated linoleic acid (CLA) refers to a group of positional and geometrical isomers of linoleic acid (1). These naturally occurring fatty acids are found in beef and dairy products due to ruminal isomerization of linoleic acid (2). Recent discoveries have shown that CLA has anti-carcinogenic activity (1), anti-atherosclerotic activity (3) and alters body composition by reducing body fat and increasing lean body mass (4). CLA was also shown to modulate the immune response by increasing lymphocyte cytotoxic activity, macrophage killing ability (5, 6) and lymphocyte blastogenesis (6, 7). Since CLA modulates immune reactivity, will it predispose animals prone to immune disorders to disease?

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease caused by defects in immune regulation that result in hyperactive T and B lymphocytes. Unlike organ-specific autoimmune diseases, a systemic autoimmune disease causes widespread tissue damage by cell-mediated immune responses, autoantibodies or immune complexes. A hallmark of SLE pathogenesis is the presence of serum autoantibodies against nuclear components as a result of immune dysregulation. For example, IgG autoantibodies to DNA are responsible for the formation of immune complexes in SLE glomerulonephritis (8, 9). The immune complexes are deposited along the wall in the small blood vessel of kidney, resulting in glomerulonephritis. Glomerular leakage of plasma proteins makes proteinuria an indicator of kidney damage originated from autoantibodies. Researchers have shown in SLE patients and rodent models that n-3 fatty acids and decreased calorie intake were beneficial in alleviating the clinical signs of disease (10-13).

Due to CLA's ability to modulate immune system, the evidence of fatty acid involvement in autoimmune disorders and the increased availability of highly enriched CLA supplements, it seemed imperative to determine the influence of CLA on autoimmune disorders. The objective of this research was to determine

the influence of dietary CLA on immune related disorders in the SLE-prone NZB/W F1 mice.

RESEARCH DESIGN AND METHODS

Materials. Conjugated linoleic acid (Natural Lipids Inc, Hovdebygda, Norway) contained 90% CLA (CLA-90) with the following C18:2 isomer distribution: 43.5% t10,c12, 41.9% c9,t11 and t9,c11, 1.5% t9,t11 and t10,t12, 0.9% c9,c11, 0.9% c10,c12. Other fatty acids in CLA-90 were 5.6% oleate, 1.4% palmitate, 0.5% linoleate, 0.4% stearate, and 3.4% unidentified compounds. All other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, Missouri) unless specified.

Diet. Semi-purified powdered diet (TD94060, 99% basal mix, Harlan-Teklad, Madison, WI) was mixed with 0.5% oil (either CLA or corn oil) and 0.5% sucrose by weight, such that the final diet contained either 5.5% corn oil (control group) or 5% corn oil plus 0.5% CLA (CLA group) (4). Diets were prepared fresh every other week and stored at 4 ^{o}C. Diets and water were provided *ad libtum*.

Animals. NZB/W F1, the offspring of New Zealand Black mice and New Zealand White mice, is a well-established animal model for human SLE study (9). Twenty female NZB/W F1 mice were obtained at four weeks of age from Harlan-Sprague Dawley (Madison, WI) and housed in a temperature and humidity controlled room with 12 hour light/dark cycle. Two mice were housed in a cage and given a pelleted chow for a week before they were randomly assigned to treatment diet (10 per diet). Protocols for animal care and use were approved by the Research Animal Resources Center of University of Wisconsin-Madison.

Feed Intake and Body Weight. Diet intake was recorded every other day in the first three weeks of the trial to determine intake and efficiency of conversion of

diet into body mass (feed efficiency). Weekly body weight and survival of the mice were also recorded from the beginning of the experiment to the time of death. For humane reasons, mice were euthanized when they became lethargic and stopped eating.

Urine Collection and Proteinuria Assay. Mouse urine was collected by using metabolic cages every other week before 28 weeks of age and weekly thereafter. Each mouse was housed in a single metabolic cage for 3 hours to collect urine. A 20 μl urine sample was diluted four times with distilled water. In a 96 well plate, 20 μl of diluted urine sample was placed in a well, and 200 μl of diluted Bio-Rad protein assay reagent was then added to each well. Plates were mildly shaken for 5 min and the color reactions were read at 600 nm with a microtiter plate reader (Autoreader EL310, Bio-tek Instrument). Duplicate samples were applied in microtiter plates and bovine γ-globulin was used as positive control and also to create a standard curve.

Serum Collection and ELISA for Anti-DNA Antibodies. Blood was drawn retro-orbitally every other week after mice were placed on their dietary treatments. Serum was separated by centrifugation and stored at -70 ^{0}C until assayed.

Enzyme-linked immunosorbent assay (ELISA) for serum anti-single strand (ss) or double strand (ds) DNA Antibody (Ab) was determined as follows: Immulon II HB plates (Dynex, Chantilly, VA), were coated with 50 μl ds calf thymus DNA (50 μg/ml) in coating buffer (50 mM sodium bicarbonate, pH 8.5). Plates were gently shaken for one hour and placed on the bench top overnight. Plates were then washed with phosphate buffered saline (PBS)-tween 20 (8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4 and 0.5 ml Tween 20 in one liter of deionized distilled water) solution four times. One hundred and fifty μl of 1% bovine serum albumin (BSA) in PBS was then added to the wells and gently

shaken for one hour. Plates were emptied by inversion. Serum samples were diluted 1: 80 with 1% BSA solution, and 50 µl of diluted serum was added to each well and plates were shaken for one hour. Plates were then washed 4 times and drained as described above. Horseradish peroxidase conjugated goat anti-mouse IgG (γ-chain specific detection antibody) was diluted 1: 2,000 with 1% BSA in PBS. To each well, 50 µl of diluted detection antibody was added. After one hour incubation and shaking, an extensive wash was applied to remove unbound detection antibody. One hundred and twenty-five µl of substrate solution (0.42 mM of TMB (3,3',5,5' tetramethyl benzidine) and 3.2 mM of H_2O_2 in 50 mM sodium acetate solution) was added to each well followed by gentle shaking for 0.2 hour. Fifty µl of stopping solution (0.5 M sulfuric acid) was then added to stop the enzymatic reaction. After gently shaking for 0.1 hour, plates were read by an ELISA reader at dual wavelength of 450 and 600 nm. Two-fold dilution of positive control serum from 1: 100 to 1: 3200 with 1% BSA was applied to each plate. Relative negative controls were also included within each plate which included normal serum control (replaces serum sample with serum from non-autoimmune mice), detection antibody control (no sample antibody added), plate control (no DNA coating and sample antibody) and substrate control (no DNA coating, sample antibody and detection antibody). Diluted serum samples were compared with in-plate positive control antibody to determine positive anti-DNA antibody ratio. In each ELISA plate, a serial dilution of positive control serum was applied and the reading of half the 1600 x dilution was arbitrarily chosen as the cut off point to determine positive anti-DNA antibody.

Statistical Analysis. Body weight, feed intake, feed efficiency and survival days were compared by Student's t-test to determine the treatment effect. Positive proteinuria ratio and positive anti-DNA IgG antibody ratio were analyzed by Fisher's exact test using a SAS computer program version 5.

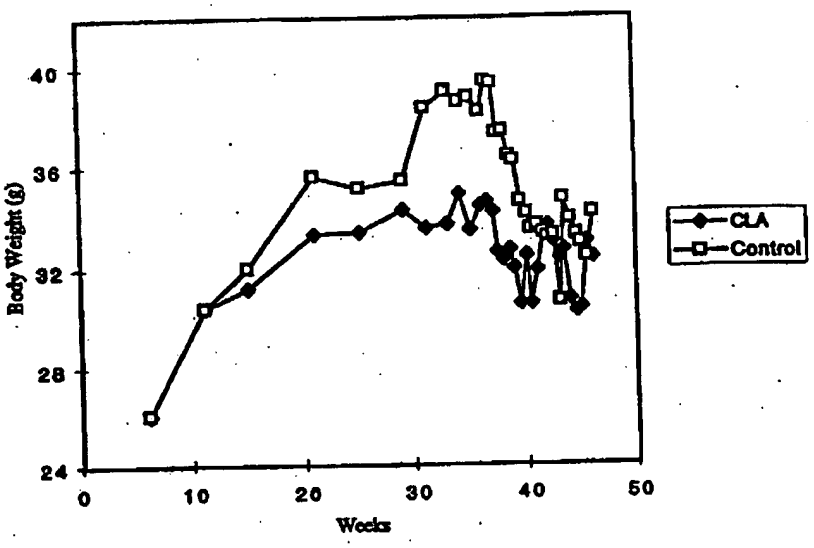


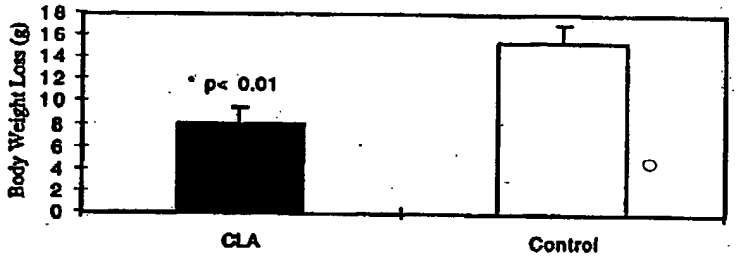
Fig. 1. Effect of dietary CLA on body weight. Body weight was not significantly different between two dietary treatments until 31 week old. Significant differences ($p < 0.05$) between CLA and control groups observed between week 31 to 39. After 39 weeks, no differences were observed. CLA = mice fed 0.5% CLA supplemented diet. Control = mice fed 0.5% corn oil supplement diet.

RESULTS

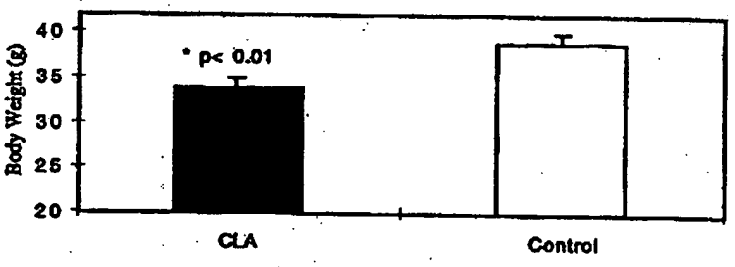
Body Weight and Food Intake. Dietary treatments had no influence on food intake for the first three weeks (CLA 161.4 g, control 166.2 g). Body weight was not affected by CLA feeding until 31 weeks of age, when the control group was found to be significantly heavier than the CLA group (Fig. 1). These differences continued until mice were 39 weeks of age, but not thereafter due to weight loss in control fed mice.

Even though control fed mice were heavier than CLA fed mice during weeks 31 to 39 (Fig. 1), dietary CLA reduced weight loss after the onset of proteinuria (Fig. 2a). The control group was heavier in body weight than the CLA group at

a. Mean body weight loss between proteinuria onset and death



b. Mean body weight at proteinuria onset



c. Mean body weight at last measure before death

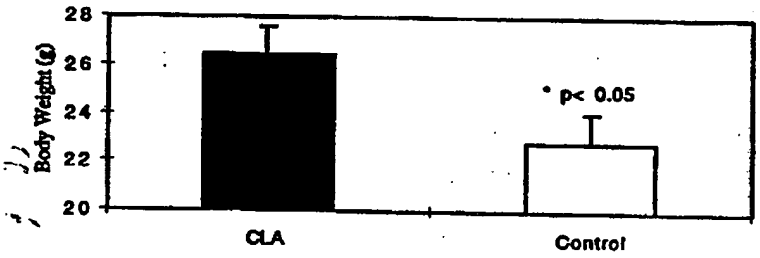


Fig.2. Effect of dietary CLA on body weight, body weight changes between proteinuria onset and death. Data are presented as Mean + SEM. SEM: Standard error of each of above means; standard error based on pooled estimate of variance from ANOVA. In (a) and (c), sample size for CLA and control groups are 7 and 6 respectively. The treatment effect is significant at $p < 0.01$ for (a) and $p < 0.05$ for (c). In (b), sample size for CLA and control groups are 10 and 8 respectively. The treatment effect is significant at $p < 0.01$. CLA = mice fed 0.5% CLA supplemented basal diet. Control = mice fed 0.5% corn oil supplement diet.

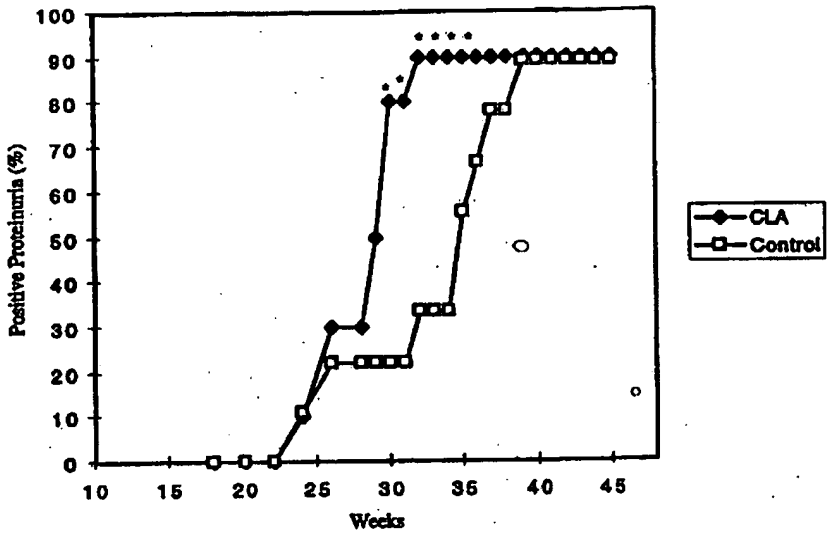


Fig 3. Effect of dietary CLA on the incidence of positive proteinuria in NZB/W F1 mice. Positive proteinuria was determined as more than 1000 μg/ml of protein in urine as described in Research Design and Methods. CLA = mice fed 0.5% CLA supplemented diet. Control = mice fed 0.5% corn oil supplement diet. * $p < 0.05$ when compared between CLA and control groups at a specific time.

the onset of proteinuria (Fig. 2b). However, by the time the disease had progressed to death, the CLA group was significantly heavier than the control group (Fig. 2c). Body weight loss after proteinuria onset until death in the control group was twice that of the CLA group (Fig. 2a). Body weight loss post proteinuria onset was not a consequence of shorter duration for CLA group. Actually, survival days after proteinuria onset was 49% longer for the CLA (88 days) than the control group (59 days) ($p < 0.05$).

Proteinuria. A minimum concentration designation of 1,000 μg/ml of urine protein was chosen to be the criteria of positive proteinuria (11, 14). The basal level of urine protein concentration was about 350 μg/ml in the mice prior to the

presence of anti-DNA antibodies. CLA fed mice had a significantly greater incidence of proteinuria than control fed mice between weeks 27 to 35, but not thereafter ($p < 0.05$) (Fig. 3).

Anti-DNA Antibodies. The percentage of mice with positive anti-ds DNA IgG antibodies was not significantly different between the groups except at week 14 when the CLA group had a higher percentage of positive serum anti-dsDNA Ab (Fig. 4a). Positive serum for anti-single stranded DNA IgG antibodies (anti-ssDNA Ab) appeared earlier than dsDNA Ab but was not influenced by dietary treatment (Fig. 4b).

Survival Rate and Day. The average life spans of CLA (296 days) and control (302 days) groups were not significantly different. However, percent survival was lower in the CLA group at some points between day 260 to day 320 (Fig. 5).

Summary of the Effect of CLA on NZB/W F1 Mice in the Development of Autoimmunity. The time line in Figure 6 shows that the CLA group tended to develop anti-ss DNA and anti-ds DNA antibodies before the control fed mice. Proteinuria onset occurred significantly earlier in the CLA fed group than the control group. While no difference was observed in the average days of survival (CLA: 296 days, control: 302 days), days of survival post onset of proteinuria was significantly (49 %) longer in the CLA fed mice when compared to the control fed mice (Fig. 6).

DISCUSSION

Conjugated linoleic acid has been shown to enhance lymphocyte blastogenesis (6, 7), and enhance select immunoglobulin production (15). Immune modulating

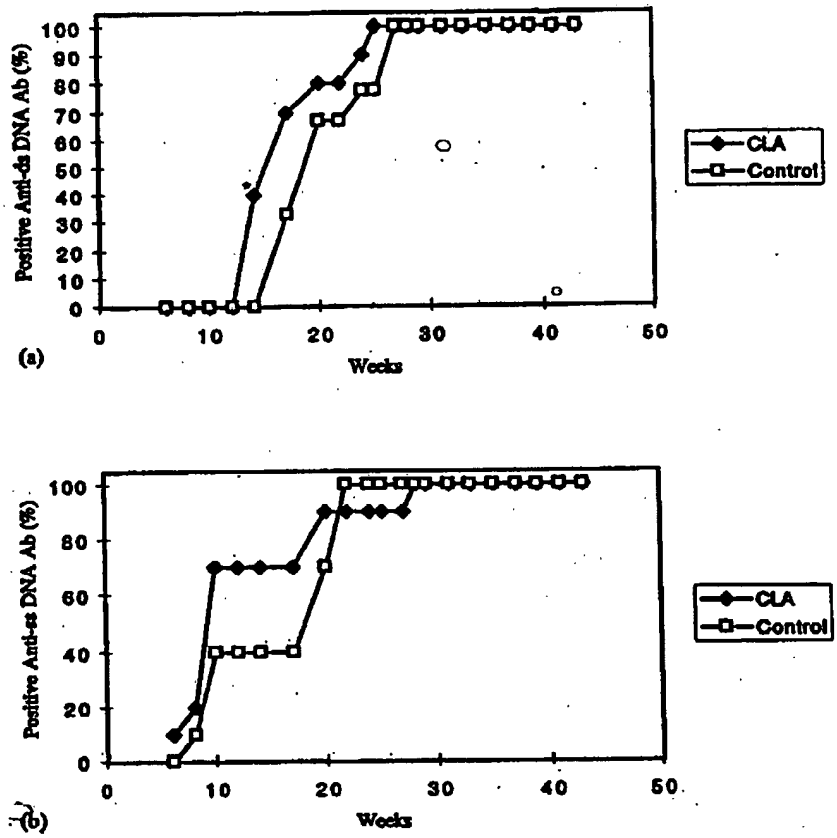


Fig. 4. Effect of dietary CLA on the percentage of positive anti-DNA antibody. Positive antibody titer was determined by comparing each sample with in-plate positive control antibody. In each ELISA plate, a serial dilution of positive control serum was applied and the reading of half the 1600 x dilution was arbitrarily chosen as the cut off point to determine positive anti-DNA antibody. Serum samples were diluted 80 x, and all duplicate samples from the same time point were run in the same plate (29, 30). (a) Positive anti-ds DNA antibody percentage (b) Positive anti-ss DNA antibody percentage. CLA = mice fed 0.5% CLA supplemented diet. Control = mice fed 0.5% corn oil supplement diet. * $p = 0.054$

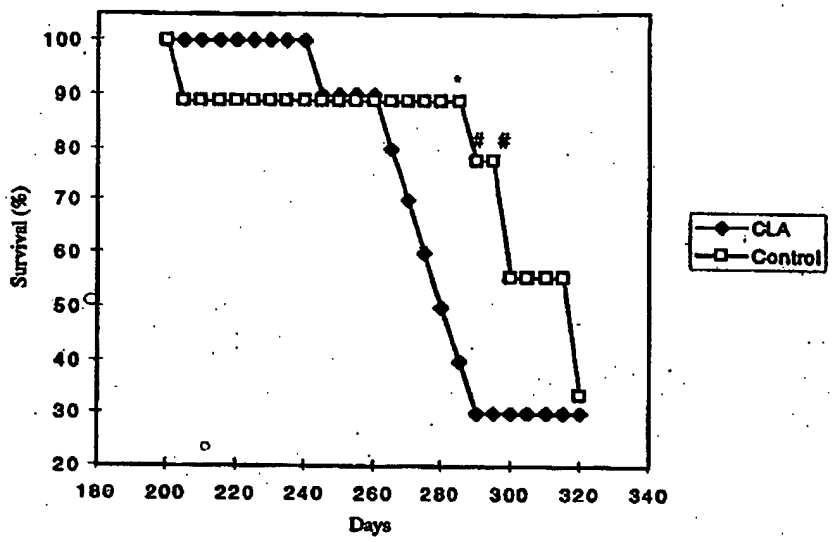


Fig. 5. Effect of CLA on survival rate in NZB/W F1 mice. CLA = mice fed 0.5% CLA supplement diet. Control = mice fed 0.5% corn oil supplement diet. * $p = 0.04$, # $p = 0.051$.

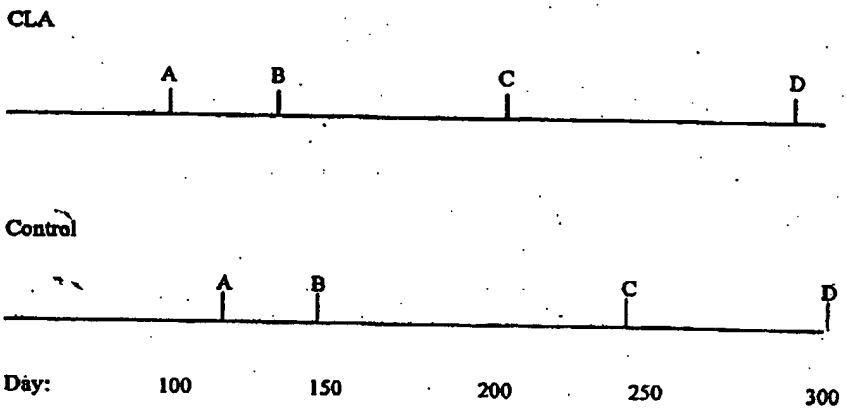


Fig. 6. Summary of the effect of CLA on NZB/W F1 mice in the development of autoimmunity. A: the average day of positive anti-ss DNA Ab (CLA: 99, control: 115). B: the average day of positive anti-ds DNA Ab (CLA: 130, control: 149). C: the average day of positive proteinuria (CLA: 208, control: 242). D: the average day of survival (CLA: 296, control: 302). Proteinuria showed up faster in CLA group than in control group, however, CLA group had a longer survival after proteinuria onset ($p < 0.05$).

activity of CLA would suggest that it would alter the events of autoimmune disease. However, in this study, NZB/W F1 mice, prone to develop anti-nuclear antibodies and succumb to renal failure, presumably to immune complexes, were not more susceptible when fed CLA than control fed mice. Another immune enhancing nutrient, α-tocopherol, showed that immune modulating abilities of a nutrient do not necessarily predict the course or nature of autoimmune disease (16). Vitamin E fed in excess of the level to prevent deficiency has been shown to enhance both humoral and cell-mediated immunities (17). When supplemented to lupus patients, vitamin E did not aggravate the autoimmune symptom (18). On the other hand, omega-3 fatty acids attenuated or delayed the onset of autoimmune disease in NZB/W F1 mice (10). These examples, and the one provided in this study, suggest that the immune modulating activity of a compound does not necessarily predict the outcome of an autoimmune disorder.

Conjugated linoleic acid did result in the production of anti-nuclear antibodies and proteinuria at an early period of life. However, the appearance of renal failure (hence death) was not accelerated in time. Actually, the time between the development of proteinuria and death was longer in the CLA fed mice than the control fed mice. These results suggest that CLA may actually protect the autoimmune mice from renal failure associated with immune complex disorders.

The reduced body weight in the CLA group relative to the control group was consistent with the data of others (19). However, CLA-fed mice lost less weight between the onset of proteinuria and death than control-fed mice. Body weight at the time of death was therefore greater in CLA-fed mice than control-fed mice. These results are not explained by the length of time between proteinuria and death, since CLA-fed mice lived an average of 22 days longer post proteinuria onset than control fed mice. The extended length of life after proteinuria in CLA-fed mice may be related to CLA's protection against end-stage autoimmune-related emaciation. CLA has been shown to protect against immune induced

cachexia (5, 7, 20). Mice, chicks, and rats lost significantly less weight post endotoxin injection when fed CLA as compared to those receiving supplemental linoleic acid. A possible mechanism by which CLA prevents immune related cachexia is through altered lipoxygenase and cyclooxygenase products (5). Decreased prostaglandins and leukotrienes in animal tissues from CLA fed animals has been demonstrated (21, 22).

CLA protection against weight loss may be the result of the reduction in macrophage cytokine production associated with dietary CLA (23). Tumor necrosis factor (TNF)-α, a potent macrophage cytokine, plays a major role in causing extensive body weight loss in chronic inflammation (24). Lower production of TNF-α has been shown in NZB/W F1 mice. Regular injections of TNF-α in NZB/W F1 retarded the onset and reduced the severity of glomerulonephritis (25). While a decreased TNF-α production or a decreased response to TNF-α (26) caused by dietary CLA provides a reasonable explanation for protection against body weight loss, despite exacerbation of autoimmune disease, it fails to explain prolonged survival post proteinuria onset.

In SLE patients, Th1/Th2 cytokine imbalance has been reported with abnormally high Th2 cytokines and low Th1 cytokine activity (27). In animal models, more complete studies have been done to show the relationship between SLE disease progression and cytokine profile change. The development of experimentally inducible SLE in mice seems to involve two stages: increased Th1 cytokines followed by increased Th2 cytokines. The increased Th1 cytokines might be important to disease induction while the increased Th2 cytokines production correlates well with disease progression to the end stage of the disease (28). Sugano et al. (22) showed dietary CLA increased IgA, IgG and IgM in both mouse serum and cultured lymph node cells, while IgE was reduced. An immunoglobulin class switch from IgM to IgG1 then to IgE requires IL-4, a potent Th2 cytokine. Their work suggested CLA enhanced Th1 cytokine production and

caused the Th2 cytokines to be inhibited. CLA's potential effects on cytokine profile may have played a role in extending days of survival post proteinuria onset. Study of such a hypothesis appears warranted.

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Dietary Conjugated Linoleic Acid Decreased Cachexia, Macrophage Tumor Necrosis Factor-α Production, and Modifies Splenocyte Cytokines Production[1]

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The effect of conjugated linoleic acid (CLA) on macrophage functions were studied *in vitro*, *in vivo*, and *ex vivo*. In RAW macrophage cell line, CLA (mixed isomers) was shown to inhibit lipopolysaccharide (LPS)-stimulated tumor necrosis factor-α (TNF-α) production. Two CLA isomers, c9,t11 and t10,c12, were tested on RAW cells and it was found that the c9,t11 was the isomer responsible for the inhibition of LPS-induced TNF-α production. BALB/c mice were used to determine the effect of dietary CLA on body weight wasting and feed intake after LPS injection. CLA was protective against LPS-induced body weight wasting and anorexia. Plasma TNF-α levels after LPS injection were lower in the CLA group compared with the corn oil-fed control group 2 hr post-LPS injection. In a separate experiment, 30 mice were fed a CLA-supplemented diet or a corn oil-supplemented diet for 6 weeks and peritoneal resident macrophages were obtained for measuring TNF-α and nitric oxide production after *in vitro* exposure to interferon-γ (IFN-γ) and/or LPS. TNF-α production was not found to be different in peritoneal macrophages from mice fed the dietary treatments, but less nitric oxide was produced in macrophages from CLA-fed mice upon stimulation when compared with macrophages from control-fed mice. Splenocytes were also collected from the mice fed the dietary treatments and stimulated to produce cytokines in culture. Supernatant was used to run cytokine enzyme-linked immunoabsorbant assays. Interleukin-4 (IL-4) was decreased in CLA-fed mice when splenocytes were stimulated with concanavalin A (Con A) for 44 hr; however, IL-2 and the IL-2-to-IL-4 ratio were elevated. Exp Biol Med 228:51–58, 2003

Key words: CLA; cachexia; macrophage; cytokines

A biological important role of conjugated linoleic acid (CLA) as an anticarcinogen was first reported by Pariza and Hargraves (1). It was later found that CLA had physiological properties that differed from LA. Synthetic CLA is a mixture of isomers, and it is difficult and costly to purify pure isomers, hence, until recently, most reports on the physiological effects of CLA have used mixed isomers (predominantly c9,t11 and t10,c12). Among these studies, CLA has been shown to decrease carcinogenesis (2, 3), decrease atherosclerosis (4, 5), and change body composition by increasing body protein and water content and decreasing body fat (6–8). In addition to the abovementioned properties, CLA also modulates the immune system by increasing lymphocyte blastogenesis, lymphocyte cytotoxic activity, and macrophage killing ability, as well as protecting against end-stage body wasting in autoimmune disease (9–12). Other fatty acids also affect immunity as well (13–15).

It has been shown that several dietary oils modulate macrophage function. For example, dietary fish oil decreased interleukin-1 (IL-1) release by peritoneal macrophages when compared with corn oil-fed controls (16). Macrophages are believed to be the principal sources of tumor necrosis factor (TNF)-α produced *in vivo*, and lipopolysaccharide (LPS) is the most potent stimulus of macrophages for TNF-α production. The activity of TNF-α was originally shown to kill tumor cells, but it also has a profound effect in causing body wasting, or cachexia. Direct infusion of TNF-α into rats has been shown to promote muscle degradation (17). Our laboratory has shown that feeding CLA to chicks reduces LPS-induced body weight wasting and feed intake (12, 18). Because macrophages are immune cells known to produce TNF-α upon LPS stimulation, the effect of CLA at inhibiting LPS-induced wasting could be due to decreased TNF-α production by macrophages.

T lymphocytes play an important role in the immune system. Upon activation, naive T cells (Th0) differentiate into either T helper 1 (Th1) or Th2 effector cells as they secret different cytokines and mediate very different im-

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mune responses (19). Th1 type responses are primarily cell-mediated immunity and inflammation, and Th2 cells and cytokines mediate humoral immunity. Upon activation, development of Th0 cells into either Th1 or Th2 cells depends on the local cytokine milieu. For example, in the presence of IL-12, Th1 clone development is preferred (20, 21). On the other hand, IL-4 is indispensable for Th0 cells to polarize into Th2 clones (22, 23). Moreover, Th1 and Th2 responses are cross-regulated. For example, IFN-γ, a cytokine produced by Th1 cells, inhibits IL-4 production and suppresses Th2 development (24, 25). Conversely, IL-4 and IL-10 produced by Th2 cells block differentiation of Th0 to Th1 (26, 27). Sugano *et al.* (11) showed dietary CLA increased immunoglobulin (Ig) A, IgG, and IgM in both rat serum and cultured lymph node cells, whereas IgE was reduced. This work implied that CLA may promote Th1 cytokine and inhibit Th2 cytokine production, as Ig class switch from IgG to IgE would not occur without IL-4 or IL-13, both which are potent Th2 cytokines (28, 29). In a guinea pig asthma model, allergen-induced trachea histamine production was reduced in CLA-fed guinea pigs (30). IgE-induced histamine release was enhanced by IL-5, a Th2 cytokine (31). It was our interest to test the effect of CLA on the helper T cell cytokines profile modification as suggested by previous studies.

Because macrophages are very sensitive to LPS stimulation, it seemed that macrophages can be a potential target of CLA to decrease LPS-induced responses. In this study, we investigated the effect of CLA on macrophage TNF-α production, as it was hypothesized that CLA inhibits TNF-α production by macrophages. In addition, splenocyte cytokines IL-2 and IL-4 were also measured.

Material and Methods

CLA. CLA used in the *in vivo* and *ex vivo* feeding trials was obtained from Natural Lipids Inc. (Hovdebygda, Norway) and contained approximately 90% CLA (CLA-90) with the following C18:2 conjugated isomer distribution: 43.5% t10,c12; 41.9% c9,t11 and t9,c11; 1.5% t9,t11 and t10,t12; 0.9% c9,c11; and 0.9% c10,c12. Other fatty acids in CLA-90 were 5.6% oleate, 1.4% palmitate, 0.5% linoleate, 0.4% stearate, and 3.4% unidentified compounds. For *in vitro* studies, LA was purchased from Nu-Check Prep (>99% pure; Elysian, MN). The c9,t11 CLA isomer was purchased from Matreya Inc. (Pleasant Gap, PA). The c9,t11 CLA isomer was 96.3% pure and had 2.6% of t9,t11/t10,t12 and 1.1% of other CLA isomers. The t10,c12 isomer was from Natural Lipids, and it had 92.8% of t10,c12; 1.6% of c9,t11; 1.2% of t9,t11/t10,t12; and 2.8% of other CLA isomers.

Macrophages. RAW 264.7 cells were a gift from M.W. Pariza (Food Research Institute, University of Wisconsin, Madison, WI). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) media (Life Technologies, Gaithersburg, MD) supplemented with 5% fetal bovine serum (FBS) and 1% antibiotics solution (A9909; Sigma, St. Louis, MO). Macrophages were plated in the density of 100,000 cells/well in a 24-well plate for 24 hr, followed by culture in fresh media containing fatty acid-albumin complex. The final fatty acid concentration was 100 μM. Cells were cultured with fatty acid-albumin complex for 24 hr. Fresh media (containing corresponding fatty acid-albumin complex) containing 500 ng/ml LPS (from *E. coli*, serotype 055:B5; L-4005; Sigma) in phosphorus-buffered saline (PBS) was then added for 16 hr to stimulate TNF-α secretion. Control cells were treated with PBS. Media was then collected, frozen, and subsequently analyzed for TNF-α as described below. Cell viability was checked under microscope and by total nonwashable protein measurement (cell protein concentration was measured after removing supernatants for TNF-α analysis and washing cells with PBS three times) to confirm that viability of macrophages cultured under the 100 μM fatty acid-albumin complex did not differ from macrophages cultured with albumin alone.

Preparation of Fatty Acid-Albumin Complex. To make fatty acid-albumin complexes for treating macrophages *in vitro*, 2.8 mg of free fatty acid, already dissolved in 0.5 ml of KOH (0.1 *M*), was transferred to a scintillation vial, and 4 ml of 2.5 m*M* bovine albumin PBS solution (LPS free, A-8806; Sigma) was added and gassed with nitrogen. The vial was then wrapped with foil and refrigerated overnight. The pH was then adjusted to 7.2 using 0.1 *M* NaOH solution, and the volume was brought to 5 ml with PBS. The solution was filtered using a 0.22-μm syringe filter for use in cell culture. The fatty acid concentration of the preparation was 2 m*M*.

Diet. A semipurified powdered diet was purchased from Harlen-Teklad (TD94060, 99% basal mix; Madison, WI). The diet had 5% corn oil and it was supplemented with either 0.5% CLA or corn oil and 0.5% sugar. Hence, the control diet had 5.5% corn oil, and the CLA diet had 5% corn oil and 0.5% CLA (10). Fresh diets were prepared every other week and were kept refrigerated. Fresh diet was provided to mice three times a week. Both diets and water were provided *ad libitum*.

Animals. Weanling BALB/c mice were purchased from Harlan-Sprague Dawley (Indianapolis, IN). In experiment one, 12 mice were immediately divided into two groups of six and were fed either CLA or the control diet. Three mice were housed together in a shoebox cage during dietary treatment and were then individually caged right before LPS injection (see below). In the *ex vivo* study, 30 mice were used in the study with 15 mice who were fed the control diet and 15 mice who were fed the CLA diet. Three mice were housed per cage in a temperature- and humidity-controlled room with a 12:12-hr light:dark cycle. The protocol for animal care and use was approved by the institutional animal care and use committee at the College of Agriculture and Life Sciences, University of Wisconsin-Madison.

***In Vivo* Mouse Trial Treatment.** After 6 weeks on the dietary treatments, three mice from each dietary group

were injected intraperitoneally with LPS (0.1 mg/ml in sterile PBS, 1 ml LPS solution/100 g of body weight) and the remaining three were injected with sterile PBS (injection control). Body weight and feed intake were recorded at 0, 24, 48, and 72 hr postendotoxin injection on the individually housed mice. Blood samples were obtained retro-orbitally at 0, 1, and 2 hr after LPS injection (32). Plasma samples were taken from the blood samples after centrifugation and were subsequently analyzed for TNF-α as described below.

Isolation and Culture of Mouse Resident Peritoneal Macrophages. In the *ex vivo* study, 30 mice were either fed the CLA (15 mice) or control diet (15 mice) for 6 weeks. Two or four mice were then sacrificed daily to obtain resident peritoneal macrophages and splenocytes. Peritoneal resident macrophages were collected posteuthanasia by injecting 10 ml of ice-cold DMEM media containing 10% FBS and 1% of antibiotics solution (Sigma) into the peritoneal cavities, and then recovering the fluid (33). The fluid was collected and gently laid on top of 3 ml of Histopaque 1081 (Sigma) and was centrifuged at 400*g* for 15 min at ambient temperature. Mononuclear cells were transferred to a clean centrifuge tube and were washed twice in Mg^{2+} Ca^{2+}-free PBS. Viable cell numbers, as determined by trypan blue exclusion, were greater than 95%. The cells were suspended and adjusted to 1×10^6 viable cells/ml in RPMI 1640 medium with 10% FBS and 1% antibiotics solution (Sigma). One hundred microliters of cell suspension was added to individual wells in a 96-well plate (100,000 cells/well) for 2 hr. Nonadherent cells were removed by washing the monolayer twice with fresh medium. The adherent peritoneal exudate cells are hereafter referred to as macrophages. Macrophages were immediately primed with 5 unit/ml IFN-γ for 4 hr before being stimulated for TNF-α or nitrite production (33). Macrophages were cultured with LPS (500 ng/ml) for 16 hr and the supernatants were collected for TNF-α assay. For nitric oxide assay, phenol-red free DMEM was used, and 500 ng/ml LPS and 5 unit/ml IFN-γ were added to the macrophages and incubated for 44 hr before the culture supernatants were removed for nitrite assay (33).

Splenocyte Isolation. Spleens were removed from the CLA- or control-fed mice and were placed in a petri dish with 10 ml of RPMI 1640 media. A 10-ml sterile syringe plunger was used to disperse the spleen into a single-celled suspension. The media containing the suspended splenocytes were then collected and layered atop 3 ml of Histopaque 1081 (Sigma) in a 15-ml centrifuge tube and were centrifuged at 400*g* for 15 min at ambient temperature. Mononuclear cells at the interface were collected using a transfer pipette, placed in another centrifugation tube, and 10 ml of media was added. Cells were centrifuged and the pellet was then washed twice with fresh media by centrifugation. Splenocytes were then resuspended to a cell density of 2×10^6/ml, and 500 μl was applied into each well in a 24-well plate. Concanavalin A (Con A) at 4 μg/ml was added into splenocyte culture, and supernatant was collected after 48 hr and frozen for subsequent cytokine analysis.

Cytokine Assay. TNF-α-, IL-2-, and IL-4- OptEIA enzyme-linked immunoabsorbant assay (ELISA) kits (PharMingen, San Diego, CA) were used to determine the serum TNF-α level as well as culture supernatant IL-2, IL-4, and TNF-α concentration. A standard procedure of cytokine ELISA was performed according to the manufacture's instructions. Briefly, a plastic plate was coated overnight with a capture antibody for a specific cytokine, followed by washing and blocking the plate. Diluted samples and standards (provided in the kit) were then added and incubated. An extensive wash was applied before secondary antibody and enzyme conjugates were added. Plates then went through another round of incubation and extensive washing. Developing reagent was then added to the plate for 15 min. Color development was stopped during linear increases in substrate utilization by adding 0.5 *M* sulfuric acid to disrupt enzymatic activity. OD readings of samples were converted to concentration based on the reference curve. At lease duplicate samples were analyzed for each cytokine ELISA. The TNF-α kit used in the present study only detects free-form TNF-α but not receptor-bound form. The detection limit for TNF-α, IL-2, and IL-4 is 20, 10, and 10 pg/ml, respectively.

Quantification of Nitrite in Culture Supernatant. Measurement of nitrite has been commonly used as an indirect measurement of nitric oxide formation because nitrite is a stable end product of the highly reactive nitric oxide (34). In the *ex vivo* experiment, 50 μl of cultured resident macrophages supernates was taken from each well and mixed with 50 μl of Griess reagent [1 part of 1% sulfanilamide and 1 part of 0.1% N-(1-naphthyl) ethylenediaminedihydrochloride, mixed immediately before use] in a different 96-well plate. The plate was gently shaken at ambient temperature for 5 min, and OD was measured at 562 nm. A standard curve was established using known $NaNO_2$ preparations. Sample nitrite concentration was extrapolated from the OD based on the standard curve. Duplicate samples were used in every nitrite microplate assay. The typical standard curve had a correlation coefficient of more than 99% between 0 and 20 μM. Samples were diluted to this range and were analyzed again if they were found to be higher than the upper limit of standards at first analysis.

Colorimetric Tetrazolium Assay. The linear relationship between number of macrophages or splenocytes and cleavage of tetrazolium was measured by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) assay (35). MTT was dissolved in PBS (5 mg/ml) and filter sterilized through a 0.22-μm syringe filter. Twenty-five microliters of MTT solution was added to each culture well and these plates were incubated at 37°C for 3 hr. One hundred microliters stop reagent was added to each well and mixed. Stop reagent was prepared by dissolving sodium dodecyl sulfate 20% (w/v) at ambient temperature in 50% N,N-dimethylformamide in demineralized wa-

ter, and pH was adjusted to 4.7 using 80% acetic acid and 2.5% 1 *N* HCl. The plates were incubated overnight at 37°C to ensure the complete solubilization of cells and the blue crystals of formazan. OD was read on a microplate reader at wavelength of 562 nm. MTT values were determined after Con A stimulation and were used to correct IL-2 and IL-4 data based on cell numbers.

Statistical Analysis. Both nonrepeated and repeated data were analyzed to determine CLA treatment effect by PROC MIX in SAS computer program, version 8 (SAS Institute, Cary, NC) (36). For Figures 1 and 2, data were analyzed by one-way analysis of variance (ANOVA) with two treatments, with repeated measures on experiment unit (mouse). A type "ARH(1)" error structure was used to account for auto-correlated errors. For Figures 3 and 4, data were analyzed by one-way ANOVA with diet as treatment and mice(diet) as error. In addition, the model included a blocking factor to account for variations due to different days of animal sacrifice. For Figure 5, data were analyzed by randomized complete block design with experiment unit

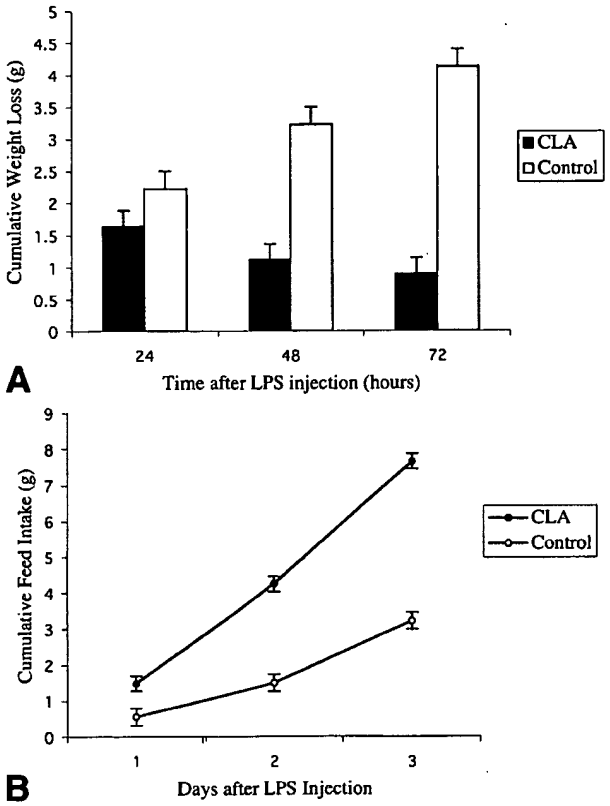

Figure 1. The influence of dietary CLA on LPS-induced body weight loss and feed intake. After feeding CLA or control diet for 6 weeks, mice were injected with either LPS (1 mg/kg) or PBS (data not shown). Cumulative body weight change and feed intake were monitored for 3 days. Each point represents least square mean with pooled error. There were three mice in each group. (a) Repeated data analysis showed dietary CLA was protecting mice against weight loss compared with mice fed the control (corn oil) diet ($P < 0.01$). (b) CLA-fed mice ate more diet after LPS injection compared with control-fed mice ($P < 0.01$).

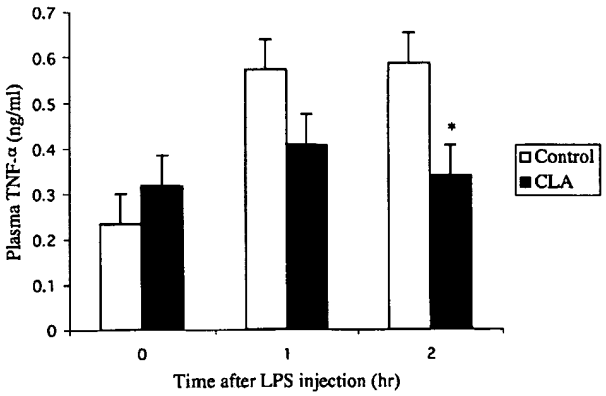

Figure 2. Influence of dietary CLA on LPS-induced release of plasma TNF-α. Mice were fed a CLA or control diet for 6 weeks before LPS (1 ng/g body weight) or PBS injection. Blood samples were obtained from mice 0, 1, and 2 hr after LPS injection. ELISA was used to determine plasma TNF-α level. Each bar represents least square mean + pooled error (SEM). Plasma TNF-α was unchanged over time in the PBS-injected mice (data not shown). An asterisk indicates significantly different from control at $P < 0.01$.

was mean of quadraplicate wells. Data from five independent experiments were used in the analysis where experiment was treated as block.

Results

Mice fed CLA had significantly less endotoxin-induced body weight loss (0.9 g) over 3 days compared with control-fed endotoxin-injected mice (4.1 g; Fig. 1A). Feed intake after LPS injection was also significantly affected by CLA. After LPS injection, CLA-fed mice ate more feed (2.6 g/day) compared with control-fed mice (1.1 g/day) over a 3-day period (Fig. 1B). In PBS injection treatment, feed intake was 2.8 g/d for the CLA group and 2.6 g/d for the control group. Weight loss in PBS injection treatment was 1.7 g in the control group and 0.1 g in the CLA group (data not shown). In the *in vivo* trial, CLA had a potent inhibitory effect on LPS-induced anorexia and body weight loss.

The evidence that CLA protects against LPS-induced cachexia and the role of dietary CLA on LPS-induced plasma TNF-α was investigated. After mice were fed CLA for 6 weeks, the plasma TNF-α levels were significantly lower in the CLA-fed mice compared with the control-fed mice 2 hr after LPS injection (Fig. 2). The TNF-α production from PBS injection control was unchanged (data not shown).

In the *ex vivo* trial, we studied peritoneal resident macrophage activity after mice were fed the dietary treatments for 6 weeks. We found that even though CLA decreased plasma TNF-α level *in vivo*, CLA did not statistically decreased LPS-induced release of TNF-α in peritoneal resident macrophage in this *ex vivo* system (Fig. 3A). Nitric oxide production, however, was reduced by CLA. Peritoneal resident macrophages from CLA-fed mice had less nitric oxide production when stimulated by IFN-γ and LPS when compared with cells from control-fed mice (Fig. 3B).

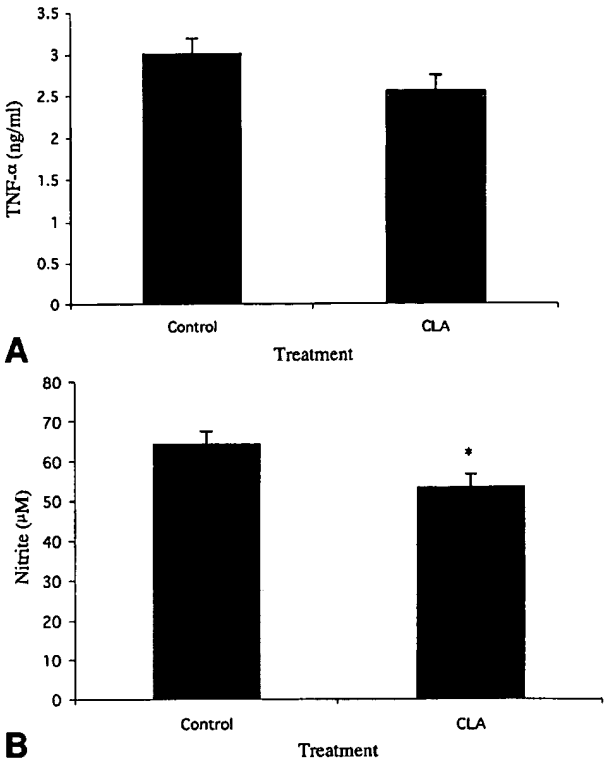

Figure 3. Nitric oxide and TNF-α production by resident peritoneal macrophages were affected by CLA feeding. Mice were fed CLA or control diet for 6 weeks before sacrifice. Resident peritoneal macrophages were obtained and primed with IFN-γ. (a) Macrophages were later stimulated with LPS for TNF-α production. (b) Macrophages were later stimulated with LPS and IFN-γ for nitric oxide production. Macrophages from mice fed a CLA diet produced a significantly lower amount of nitrite (53.3 μM) compared with macrophages from mice fed a control diet (64.2 μM). TNF-α production, however, was not different between CLA and control groups. Each bar represents least square mean with pooled error. There were 15 mice in each diet treatment. An asterisk indicates significantly different from control at $P < 0.05$.

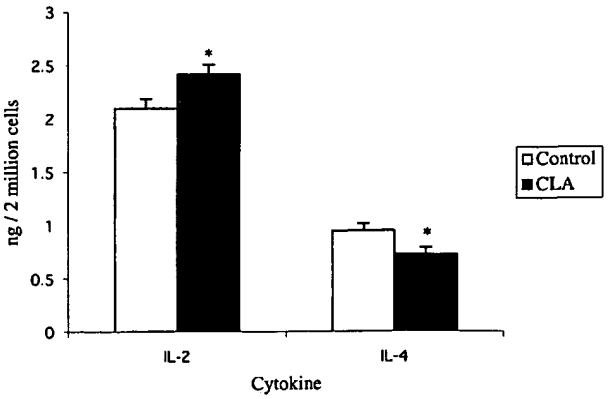

Figure 4. Cytokine production by splenocytes from mice fed CLA or control diet. After mice were fed CLA or control diet for 6 weeks, splenocytes were harvested and stimulated with concanavalin A for cytokine production. Supernatants were then harvested from the cultured splenocytes and analyzed by ELISA. Cytokine production from each well was corrected by numbers of cells presented at the end of the culture that was determined by colorimetric tetrazolium assay as described in "Materials and Methods." Each bar represents least square mean with pooled error. There are 15 mice in each diet treatment. The IL-2-to-IL-4 ratio is 3.3 for the CLA group and 2.2 for the control group. An asterisk indicates significantly different from control at $P < 0.05$.

Spleens were also obtained for lymphocyte cytokine analysis. Splenocytes were treated with Con A for 48 hr before collecting supernatant for cytokine analysis. Splenocytes from CLA-fed mice did show a higher IL-2 production after LPS stimulation and lower IL-4 production than splenocytes from control-fed mice (Fig. 4). The ratio of IL-2 to IL-4 was significantly ($P < 0.05$) higher (ratio = 3.3) for splenocytes from the CLA-fed mice compared with the control-fed mice (ratio = 2.2).

In the *in vitro* experiment, after RAW macrophages were cultured with fatty acid-albumin complexes, they were stimulated with LPS to produce TNF-α. Our data showed that CLA mixture had an inhibitory effect on TNF-α production compared with the LA control. Among isomers, the c9,t11 isomer was responsible for this inhibitory effect (Fig. 5A), but not the t10,c12 isomer (Fig. 5B). In average, 100 μ*M* c9,t11 CLA isomer decreased macrophage TNF-α production by 60% compared with LA control. This c9,t11 isomer effect was dose responsive as 50 μ*M* c9,t11 CLA alone was 40% inhibitory to macrophage TNF-α production. On the other hand, t10,c12 isomer did not show any effect on macrophage TNF-α production.

Discussion

Dietary fatty acids have been shown to be potent immune regulators. In general, high dietary levels of PUFA or n-3 fatty acids have an inhibitory effect on T cell proliferation and natural killer (NK) cell activity (37). Fish oil, rich in n-3 fatty acids, decreased murine and human LPS-stimulated TNF-α production (38, 39). It has been shown that dietary fish oil prevents endotoxin-induced death in guinea pigs (40), possibly by inhibition of cytokines produced by macrophages (41).

Feeding 0.5% CLA to animals resulted in up to 10 mg of CLA per gram of body fat in several different tissues within 30 days (42), and it took between 2 and 4 weeks to reduce CLA to baseline levels after its withdrawal from the diet (43). Several physiological changes, including body composition change (6), anticarcinogenesis (44), and antiatherosclerosis (4), were also shown when using up to 0.5% CLA in animal studies.

CLA has been shown to be a potent immune regulator. CLA decreased the LPS-induced cachectic response in chicken, rats, and mice, but at the same time, increased lymphocyte blastogenesis (12, 18). Other groups also reported CLA-increased lymphocyte blastogenesis as well as increased IL-2 production (9, 45, 46). To the best of our knowledge, this is the first report of an elevated Th1/Th2 cytokine ratio in CLA-fed mice. The increased IL-2 production (shown here, and by others) and the decreased IL-4 production associated with dietary CLA may explain the

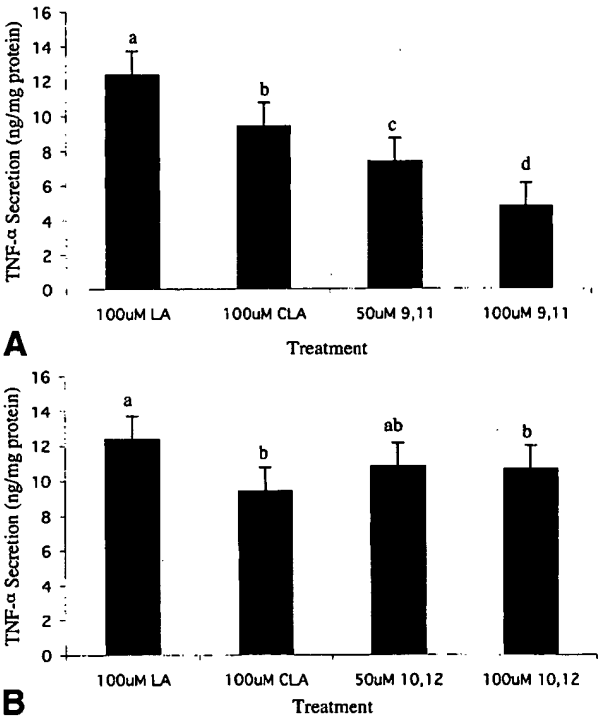

Figure 5. CLA inhibited TNF-α production from cultured RAW macrophage cell line (a and b). RAW cells were plated (100,000 cells/well) in 24-well plate for 24 hr. Then they were cultured with fatty acid-albumin complex for 24 hr before LPS was added into culture to stimulate TNF-α secretion for 16 hr. Each bar represents least square mean with pooled error (SEM). There were 4 wells for each treatment in a 24-well plate. Bar with a different letter indicates significantly difference at $P < 0.05$. (a) Compared among LA, CLA, and c9, t11 CLA isomer. (b) Compared among LA, CLA, and t10, c12 CLA isomer. Mixed isomers, approximately 42% c9,t11 and 44% t10,c12. Figure shown here represented means of five independent experiments.

reduced IgE production in CLA fed rats (11) as compared with rats fed diets largely based on LA.

Because CLA seems to enhance immunological responses, which may pose a potential risk on immune hypersensitivity, our laboratory has been studying how dietary CLA may affect type I and type III immune hypersensitivity. In a type I hypersensitivity model, CLA reduced antigen-induced histamine and PGE_2 release from tracheae of CLA-fed guinea pigs (30). In an autoimmune (type III hypersensitivity) model, CLA feeding not only protected against end-stage body wasting, but also increased survival days 1.5-fold after the onset of proteinuria in mice (10). Shifted cytokine profiles toward Th1 may interfere with Ig class switch that is needed in producing IgE in immune type I hypersensitivity responses (47). The reduced IgE production by CLA-fed rats (11) would certainly result in a reduced type I hypersensitivity reaction upon challenge, as demonstrated in a guinea pig hypersensitivity model (30). The development of experimentally inducible lupus in mice seems to involve two stages: increased Th1 cytokines followed by elevated Th2 cytokines later in life (48). Increased Th2 cytokine production correlates well to disease progression. Hence, a CLA-induced shift toward Th1 cytokines could explain, at least in part, increased days of survival post-proteinuria in lupus-prone mice. Inhibited production of TNF-α may also help to explain the reduction in body weight loss in CLA-fed lupus mice at the end stage of systemic lupus disease (10).

Macrophages are sensitive to dietary fatty acid supplementation, as its fatty acid profile reflects such dietary intervention (49, 50). These works also showed that dietary fatty acids also dictate macrophage physiology. Dietary fish oil has been shown to decrease macrophage antigen presentation (51, 52) and to decrease cytokine production as well as mRNA expression (38, 53). In the present study, macrophages were tested as one of the target cells on which CLA modulates immune function. Our data showed that CLA, more specifically c9,t11 CLA, decreased TNF-α production in RAW macrophage cell line. LPS-induced anorexia and cachexia responses were reduced in CLA-fed animals. The plasma TNF-α level after LPS injection was also suppressed in CLA-fed mice. It was unanticipated that mice fed CLA did not suppress *ex vivo* TNF-α production in resident peritoneal macrophages. However, both *in vitro* and plasma samples showed that CLA has an inhibitory effect on TNF-α production. Fatty acid turnover rate in cultured peritoneal resident macrophages may have played a role in the lack of TNF-α response observed in this model. In the cell culture study, CLA was kept at a constant concentration during the *in vitro* experiments, and when blood was drawn for TNF-α analysis, mice were still fed the treatment diet. However, when culture supernatants were collected in the *ex vivo* study, peritoneal macrophages had already been cultured *in vitro* for 24 hr without exogenous exposure to CLA. Moreover, the fetal bovine serum used in the system might not have been as effective as autologous serum (15) in demonstrating CLA's effects on TNF-α production by resident peritoneal macrophages.

The two main isomers of the CLA mixture (c9,t11 and t10,c12) used in this study were considered to have different properties on cytokine regulation. In fact, c9,t11 CLA increased feed efficiency (54, 55), whereas the t10,c12 was the isomer shown to affect body composition (56). In the present study, c9,t11 CLA isomer inhibited TNF-α production from *in vitro* macrophage cultures (Fig. 5a). The slight decrease of TNF-α in 100 μM t10,c12 CLA isomer treatment probably was thought to be due to c9,t11 CLA isomer contamination of the t10,t12 CLA isomer used (Fig. 5b). Moreover, a recent study reported decreased hepatic TNF-α mRNA expression by c9,t11 CLA in mice (57). It remains to be shown which isomers downregulate IL-4 production and increase IL-2 production. Even though the *in vitro* data suggested that c9,t11 CLA is the active isomer in reducing LPS-induced TNF-α production, further *in vivo* studies with pure CLA isomers are required to validate the *in vitro* data on TNF-α production and determine the effects of different CLA isomers on immune function.

TNF-α was shown to be involved in endotoxin-induced

weight loss and cancer cachexia (58). Muscle degradation was enhanced by TNF-α. Blocking TNF-α function by either injection of anti-TNF-α antibody or TNF-α-binding proteins reduced the cachexia reaction (59, 60). The decreased LPS-induced TNF-α production by CLA may provide at least one mechanism by which CLA enhanced the rate of growth and improved feed efficiency in animals (12, 54, 55).

Thromboxane A_2 (TXA_2) is a mediator of renal damage (61). In murine lupus, TXA_2 inhibitor prolonged survival of NZB/W F1 mice, and renal TXA_2 production was elevated in F1 mice (62). In a study comparing TXB_2 (stable end product of TXA_2) production and COX gene expression of peripheral blood mononuclear cells (PBMC) among patients with active or inactive lupus nephritis and healthy individuals as a control, COX-2 expression and TXB_2 production in PBMC was only elevated in patients with active lupus nephritis (63). Immunostaining of kidney biopsies showed no difference in COX-1, but COX-2 staining in patients with active lupus nephritis was increased compared with patients with inactive lupus nephritis or healthy controls. Double staining of kidney biopsies with anti-COX-2 and anti-CD68 antibodies (macrophage marker) demonstrated that upregulated COX-2 enzyme expression was within the macrophages of glomeruli in patients with active lupus nephritis (63).

Infiltrating macrophages in glomeruli, with upregulated COX-2 enzyme expression, may contribute to the elevated TXA_2 level in active lupus nephritis. Regulation of macrophage activities and eicosanoid profiles by CLA may play a role in lupus nephritis. Because COX-2 is the inducible form and the COX-1 is constitutively expressed, decreased antigen-induced TXB_2 production but not the basal level of TXB_2 production in CLA-fed guinea pigs provides an explanation that CLA may preferentially inhibit COX-2 activity (64). Decreased macrophage activities including TNF-α and nitric oxide productions seen in this study may help to explain the effect of CLA in prolonging survival of NZB/W F1 mice.

In conclusion, these data suggest that cytokine regulation by CLA could be responsible for previous reports demonstrating CLA's anticachectic effects, increased lymphocyte blastogenesis, decreased immune type-1 hypersensitivity, decreased IgE production, and prolonged life post proteinuria in the NZB/W F1 autoimmune mouse.

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